EXERCISES IN PRACTICAL PHYSIOLOGICAL CHEMISTRY

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Exercises in Practical Physiological Chemistry

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SECOND EDITION

CAMBRIDGE: W. HEFFER & SONS
LONDON: SIMPKIN, MARSHALL & Co.
1908

PREFACE
TO THE FIRST EDITION.

My aim in writing this book has been to present to the student a series of exercises which can be successfully carried through in ordinary class work.

Too often a student is discouraged in his work and displeased with his Text-Book by finding that a minute care in following the instruction given fails to produce the specified result. I trust that no such difficulty will be met with in working through this Book. Each and every exercise given here I have first worked through and obtained the result stated. All I ask of the student is a zealous and interested care and he will then have no difficulty in performing the experiments and learning the lessons they teach.

The ground covered is more than is at present necessary for most examinations in medicine, but I feel that this is justified by the growing importance of the subject and the increasing standard of the knowledge of it required of candidates at these examinations.

A special feature of the book is the notes that follow certain of the exercises. These notes summarise a series of exercises, indicate the special precautions that are necessary for success or give the probable reasons for an apparent failure in the performance of a given exercise. They should be carefully studied both before and after the exercise to which they refer. At the end of the book spaces are provided for the student to draw various crystalline forms from preparations made by himself. I consider this a more instructive plan than giving illustrations of typical crystals, which often differ considerably from those prepared in class work. A blank chart for recording the absorption spectra of various pigment solutions and colour reactions is also added. The drawings should be shown to the demonstrator of the class for comments or corrections.

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November, 1904.

PREFACE
TO THE SECOND EDITION.

Beyond a few minor alterations and additions the chief changes are the adoption of the new nomenclature for the proteins as recommended by the Physiological Society; a new set of exercises on the globulins of blood-serum; and new methods that have been devised for the quantitative estimation of sugar. Experience with some hundreds of students during the past four years makes me desirous of emphasising the importance of the notes that follow the exercises. If they are neglected, the work tends to degenerate into the "rule of thumb" order, which has been the standing reproach of physiological chemistry for so long.

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CAMBRIDGE,
July, 1908.

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CHAPTER I.

THE PROTEINS.

A. The colour reactions of Proteins.

For the following reactions use egg-white that has been well beaten with six times its volume of water, or serum that has been diluted ten times with water.

1. **The Xanthoproteic reaction.** To 5 c.c. of the protein solution in a test tube add about one-third of its volume of strong nitric acid. A white precipitate is formed. Boil for a minute. The precipitate turns yellow and partly dissolves to give a yellow solution. Cool under the tap and add strong ammonia till the reaction is alkaline. The yellow colour is turned to orange.

NOTES—1. The essential features of the reaction are that a yellow colour is obtained when the solution is boiled with strong nitric acid, and that this yellow colour is intensified on the subsequent addition of ammonia. The white precipitate with nitric acid is not obtained with certain proteins (See Ex. 12).

2. The reaction is given by all aromatic substances, such as benzoic acid (C_6H_5 . COOH); phenol (C_6H_5 . OH);

salicylic acid $\left(C_6H_4\begin{matrix}OH\\COOH\end{matrix}\right)$, etc.

3. The aromatic substances in the protein molecule that are responsible for the reaction are chiefly tyrosin and tryptophane.

4. Oleic acid, olive oil and most vegetable oils give a well-marked xantho-proteic reaction.

2. **Millon's reaction.**. Treat 5 c.c. of the protein solution with half its volume of Millon's reagent. A white precipitate is formed. Boil the mixture. The precipitate turns to a brick-red colour, or disappears and leaves a red solution.

NOTES—1. The essential feature of the reaction is the red colour on boiling. The white precipitate in the cold is due to the action of the mercuric nitrate on the proteins (See Ex. 14).

2. A white precipitate is also obtained with solutions of urea (See Ex. 211).

3. Sulphates give a white precipitate of mercurous sulphate.

4. The reagent is obtained by dissolving mercury in nitric acid and diluting with water. It contains mercurous and mercuric nitrates, excess of nitric acid, and a small amount of nitrous acid.

5. The reaction should never be attempted with a strongly alkaline fluid, since the alkali will precipitate the yellow or black oxides of mercury.

6. The reaction is given with all aromatic substances that contain a hydroxyl group attached to the benzene ring. Thus it is given by phenol, salicylic acid, and naphthol, but is not given by benzoic acid.

7. The aromatic substance derived from protein that is responsible for the reaction is tyrosin. $C_6H_4\begin{cases}OH & (1)\\ CH_2 . CH . NH_2 . COOH & (4)\end{cases}$

3. **The glyoxylic reaction.** (Hopkins and Cole). Treat 2 or 3 c.c. of the fluid with the same bulk of "reduced oxalic acid." Mix and add an equal volume of concentrated sulphuric acid, pouring it down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tubes gently from side to side. The purple colour spreads through the whole fluid.

NOTES—1. "Reduced oxalic acid" is prepared by treating half a litre of a saturated solution of oxalic acid with 40 grammes of 2 per cent. sodium amalgam in a tall cylinder. When all the hydrogen

has been evolved the solution is filtered and diluted with twice its volume of water. The solution now contains oxalic acid, sodium binoxalate and glyoxylic acid (COOH . CHO). It should be kept in a closed bottle containing a little chloroform.

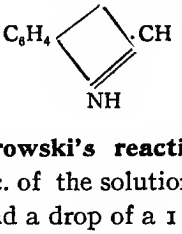
2. The reaction does not succeed in the presence of nitrates, chlorates, nitrites or excess of chlorides.

3. The colour is not well seen if the protein is mixed with certain carbohydrates (*e.g.* cane-sugar) owing to the char produced by the strong sulphuric acid.

4. It is most important to use pure sulphuric acid for this test. It sometimes fails owing to the presence of impurities in the acid employed.

5. In performing the test on a solid substance, like fibrin or keratin, a small amount of the material should be heated with a few c.c. of the reduced oxalic acid and an equal volume of strong sulphuric acid. The mixture is shaken and as the protein dissolves in the strong acid both the fluid and the solid particles assume a purple colour.

6. The substance in the protein molecule that is responsible for the reaction is tryptophane.



4. Piotrowski's reaction (the biuret reaction). Treat 5 c.c. of the solution with an excess of sodium hydrate and a drop of a 1 per cent. solution of copper sulphate. A violet or pink colour is produced.

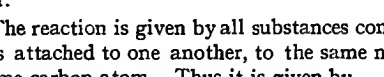
NOTES—1. The reaction is of especial importance in testing for albumoses and peptones, which give a rose colour. It is generally stated that other proteins give a violet, but usually egg-albumin gives a distinct rose tint.

2. The nomenclature of the reaction is somewhat varied. Some writers use the term "biuret reaction" even when performed on serum-proteins, but others restrict this term to the reaction given by albumoses and peptones, employing the term "Piotrowski's reaction" to that given by albumins and globulins.

3. The test cannot be applied in the presence of a large amount of magnesium sulphate, owing to the precipitation of magnesium hydrate by the alkali.

4. If the solution contains much ammonium sulphate it must be treated with a large excess of strong sodium hydrate as directed in Ex. 51.

5. The reaction is given by all substances containing two CONH groups attached to one another, to the same nitrogen atom, or to the same carbon atom. Thus it is given by



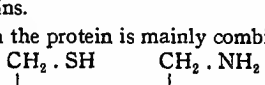
Oxamide. Biuret (See Ex. 214). Malonamide.

5. The sulphur reaction. Boil a little undiluted egg-white or serum with some 40 per cent. sodium hydrate for two minutes, and then add a drop or two of lead acetate. The solution turns deep black.

NOTES—1. This reaction is due to the fact that the sulphur of the protein is liberated as sodium sulphide when boiled with the strong alkali. The sulphide gives a black colour or precipitate of lead sulphide when the solution is subsequently treated with lead acetate.

2. The reaction does not succeed with caseinogen, peptones, and certain other proteins.

3. The sulphur in the protein is mainly combined as cystëin



B. The Chemistry of Serum and of the Serum-Proteins.

Ox blood is collected into pans and is allowed to stand till clotting is complete. The serum that exudes is pipetted off and kept in the ice chest till required.

6. Take the specific gravity by floating a clean,

dry urinometer in a cylinder containing the serum, and noting the graduation where the stem of the urinometer is level with the surface of the fluid. It is usually about 1030 (water being taken as 1000).

7. Take the reaction of the serum to litmus paper. It is alkaline.

8. Dilute 5 c.c. of serum with 50 c.c. distilled water.

(*a*) Boil 5 c.c. in a test tube. The solution sometimes becomes opalescent, but no definite coagulum is formed. Cool the tube and add 1 per cent. acetic acid drop by drop. A precipitate of meta-protein is formed, soluble in excess of acid.

(*b*) Boil 5 c.c. with two drops of 1 per cent. acetic acid. A white flocculent precipitate is formed. Cool the tube and add two or three drops of strong nitric acid. The precipitate does not dissolve.

(*c*) Boil 5 c.c. with a drop of strong acetic acid. A precipitate is not formed. Cool the tube and add 2 per cent. sodium carbonate, drop by drop. A precipitate of meta-protein is formed which redissolves in excess.

(*d*) Boil 5 c.c. with two drops of 2 per cent. Na_2CO_3. A coagulum is not formed. Cool the tube and add 1 per cent. acetic acid. A precipitate of meta-protein is formed, soluble in excess of acid.

NOTES—1. These reactions are of very great importance, and are to be explained as follows: Serum contains two varieties of proteins, known as globulins and albumins, which are coagulated by boiling, provided that the reaction of the fluid is neutral or very

faintly acid. If the solution is alkaline (and it must be remembered that serum is alkaline) the proteins are acted on by the alkali as the temperature rises and are converted to a substance known as meta-protein, which is not coagulated by heat *when in solution*. If the reaction be markedly acid, as in (*c*), the proteins are, similarly, converted to meta-protein. But if the reaction be neutral or only faintly acid, as in (*b*), a coagulum is formed on boiling. This coagulum consists of the whole of the albumin and globulin and is insoluble in water, dilute acids, and dilute alkalies.

2. The addition of the nitric acid in (*b*) is to ensure that the precipitate that appears on boiling does not consist of calcium or magnesium phosphate, which is soluble in dilute nitric acid. That such a phosphatic precipitate can be formed on boiling certain solutions is shown by the following experiment. Treat a solution of calcium chloride with sodium phosphate and then with excess of sodium carbonate. A precipitate of $Ca_3(PO_4)_2$ appears. Add acetic acid drop by drop till the precipitate just dissolves, the acid phosphate being formed. Boil the solution for half a minute. A white precipitate appears. Add a drop or two of nitric acid. The precipitate dissolves. The appearance of the precipitate of $Ca_3(PO_4)_2$ on boiling is due to the alteration of reaction as the CO_2 is evolved.

9. Take 5 c.c. of the diluted serum in a test tube: add two drops of 1 per cent. acetic acid and place the test tube in a beaker of water, supporting it by a clamp so that it does not touch the bottom of the beaker. Heat the water with a Bunsen flame and note the temperature at which coagulation begins. It usually commences at about 70°C., and is complete at 82°C. It chiefly takes place between 73° and 75°C.

NOTE.—The various albumins and globulins have different coagulating points, but since this point varies with the concentration of the electrolytes in the solution it can only be used for separating and distinguishing proteins when the conditions are similar. Nevertheless the coagulation temperature serves to distinguish myosin (56°C.) and fibrinogen (56°C.) from serum-globulin (73°C.).

10. Allow a few drops of serum to fall into about 10 c.c. of strong alcohol. A white precipitate is formed. Shake well and allow to stand for half an hour. Filter and treat the precipitate with water. It does not dissolve, showing that the serum-proteins are coagulated and not merely precipitated by strong alcohol.

11. Treat 5 c.c. of diluted serum with two or three drops of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed. Boil. The precipitate does not dissolve.

NOTES—1. Primary albumoses are also precipitated by potassium ferrocyanide and acetic acid, but the precipitate produced dissolves on warming and reappears on cooling (See Ex. 49).

2. The precipitate and fluid often become coloured a blue-green on boiling. This is due to a decomposition of the ferrocyanic acid on boiling it with certain organic substances, such as proteins.

12. To about 5 c.c. of diluted serum add a few drops of strong acetic acid. A precipitate is not formed. Now add four or five drops of strong nitric acid: a white precipitate is formed.

NOTE.—The serum-proteins are not precipitated by acetic acid (thus differing from mucin and nucleo-protein). The action of nitric acid on serum-proteins is to produce meta-protein, which is insoluble in *strong* mineral acids. This precipitability of albumins and globulins is the basis of Heller's test for these proteins in urine (Ex. 241).

13. To some serum diluted about ten times add a few drops of a solution of tannic acid. A white precipitate is formed.

14. To another portion add a few drops of a solution of lead acetate, or mercuric nitrate. A white precipitate is formed.

The remaining exercises of this section deal with the properties of the globulins and albumins of the serum. Before performing these the student should read the following account of the reactions of these proteins.

The Globulins are insoluble in distilled water, soluble in dilute acids and alkalies, and in weak solutions of neutral salts.

A neutral solution in a dilute salt is coagulated on boiling.

A solution in dilute acid or alkali is converted to a solution of meta-protein on boiling.

If the globulin be dissolved in a minimum amount of a salt and the solution be diluted with several volumes of distilled water, the globulin is partially precipitated. If the globulin be dissolved in dilute acid or alkali, there is no precipitation on dilution.

The globulins are completely precipitated by full saturation with magnesium sulphate or by half-saturation with ammonium sulphate, *i.e.*, by treatment of the solution with an equal volume of a saturated solution of ammonium sulphate.

The Albumins are soluble in distilled water, dilute salt solutions, dilute acids and alkalies.

A neutral solution in water or salt is coagulated on boiling.

A solution in dilute acid or alkali is converted to a solution of meta-protein on boiling.

Solutions of albumins are only partially precipitated by saturation with magnesium sulphate or by half-saturation with ammonium sulphate if the reaction of the solution be neutral or alkaline.

They are more completely precipitated by these solutions in the presence of acid.

They are completely precipitated by full saturation with ammonium sulphate from a neutral, acid or alkaline solution.

15. Dilute 5 c.c. of serum with 50 c.c. of distilled water. A faint cloud of serum globulin is formed. Add ·4 p.c. hydrochloric or 1 p.c. acetic acid, drop by drop. The cloud becomes denser.

NOTE.—The globulin in the serum is held in solution both by salts and dilute alkalies. Dilution alone produces a very small precipitate, but if the solution be now treated with just sufficient acid to neutralise the alkali, a much larger fraction of the globulin is thrown down.

16. Prepare a suspension of globulin by the following method. To 15 c.c. of serum in a beaker add 2 c.c. (about 30 drops) of 1 p.c. acetic acid and 100 c.c. distilled water. Stir and allow the mixture to stand for about 20 minutes. A precipitate of globulin settles down. Very carefully pour off the supernatant

fluid and divide the suspended globulin into two equal portions in clean test-tubes. With these perform the two following exercises.

17. Add a 5 p.c. solution of sodium chloride, drop by drop, till the globulin has just dissolved. Divide the solution into three portions A, B and C.

(*a*) Boil. The protein is coagulated.

(*b*) Dilute with about five volumes of distilled water. The globulin is partially reprecipitated.

(*c*) Treat with an equal volume of saturated ammonium sulphate solution. The globulin is reprecipitated.

18. Add ·4 p.c. HCl., drop by drop, till the globulin has just dissolved. Divide the solution into three portions D, E and F.

(*d*) Add 2 p.c. sodium carbonate solution till the globulin is partially reprecipitated (one or two drops only are necessary). Now add a few drops of 5 p.c. sodium chloride. The precipitate of globulin redissolves.

(*e*) Boil the solution. The protein is not coagulated. Cool under the tap and add enough 2 p.c. sodium carbonate to precipitate the meta-protein that has been formed by boiling. Now add a few drops of 5 p.c. sodium chloride. The precipitate of meta-protein does not dissolve.

(*f*) Dilute with about five volumes of distilled water. The globulin is not thrown out of solution.

19. Mix about 10 c.c. of undiluted serum with an exactly equal quantity of a saturated solution of ammonium sulphate. A thick white precipitate is formed consisting of the whole of the globulin and a portion of the albumin. Filter through a dry filter paper into a dry test tube. Label the filtrate A. Scrape the precipitate off the paper and treat it with distilled water. The precipitate dissolves, the ammonium sulphate adhering to it forming a dilute salt solution which allows the globulin to go into solution. Boil a portion of this solution with a drop of 1 per cent. acetic acid. A heat-coagulum is formed.

20. Filtrate A contains serum-albumin in the presence of half-saturated ammonium sulphate. Apply the following tests.

(*a*) Boil a portion. A heat-coagulum is formed.

(*b*) To another add one drop of strong acetic acid. A white precipitate of serum-albumin is formed.

(*c*) Grind the remainder in a mortar with solid $(NH_4)_2SO_4$ till the fluid is saturated. A white precipitate of serum-albumin is formed. Filter off the precipitate and test the filtrate for proteins either by boiling or by the glyoxylic or xanthoproteic reactions. Proteins are absent, showing that all the proteins of serum are precipitated by complete saturation with $(NH_4)_2SO_4$.

21. Serum has been dialysed in parchment tubes for two or three days against repeated changes of

distilled water. Note the heavy precipitate of serum-globulin that has fallen to the bottom of the tube.

22. Dilute 5 c.c. of serum with five times its volume of tap water, add a drop or two of 2 per cent. calcium chloride and a drop or two of neutral litmus. Boil the mixture in a porcelain dish and whilst boiling cautiously add 1 per cent. acetic acid till the reaction is *faintly* acid. Filter, and test the filtrate for proteins by the usual colour tests. If the operation has been carried out successfully the filtrate will be found to be free from proteins.

NOTE.—This is the method usually employed for removing albumins and globulins from solution. It is obvious from the note to EX. 8, that a certain amount of meta-protein is formed when the fluid is first boiled, but this meta-protein is precipitated by the acetic acid, and the precipitate is coagulated at boiling temperature so that it does not re-dissolve in the very slight excess of acid that is subsequently added (see Chapter I. D).

It is most important that the acid should be added slowly and not in any excess. The small amount of calcium chloride added aids in the aggregation of the protein on boiling.

C. The Chemistry of Egg-white.

23. In egg-white which has been well beaten with a whisk to break up the containing membranes, and diluted with four times its volume of distilled water, note a precipitate of ovo-mucin and globulin. Perform the following tests:

(*a*) Take the reaction to litmus. It is alkaline.

(*b*) Add two or three drops of saturated $(NH_4)_2SO_4$ (to prevent the precipitation of the globulin) and then two drops of strong acetic acid. Note the precipi-

tation of a protein known as ovo-mucin. Remove this by filtration, carefully neutralise the filtrate and perform the following reactions.

(*c*) Boil a portion. A coagulum is formed, indicating the presence of either a globulin or an albumin.

(*d*) Make another portion very faintly alkaline by the addition of a drop or two of 2 per cent. Na_2CO_3. Now add an equal bulk of saturated $(NH_4)_2SO_4$. A slight precipitate of globulin or albumin is formed. Filter this off, and boil a portion of the filtrate with a drop of 1 per cent. acetic acid. A coagulum of albumin is formed. Saturate the remainder of this filtrate with $(NH_4)_2SO_4$ by grinding with the solid in a mortar. A precipitate of albumin is formed.

(*e*) Completely remove the globulin and albumin by boiling. Filter and apply Millon's or the xantho-proteic protein tests to the filtrate. Protein is found in small quantities. This protein is known as ovo-mucoid. It is not coagulated by boiling, or precipitated by acetic acid. It is precipitated by saturation with $(NH_4)_2SO_4$ and also by strong alcohol.

24. **The crystallisation of egg-albumin.** (Hopkins' method). Separate the white from a number of new-laid eggs, taking care not to allow any of the yolk to mix with the white. Measure the egg-white and churn it up with an exactly equal volume of a neutral fully-saturated solution of ammonium sulphate by means of a whisk, adding the sulphate in

portions and mixing thoroughly after every addition. Notice the strong smell of ammonia that is evolved. Filter the mixture through a large pleated filter-paper. Measure the filtrate. Take 100 c.c. of it and cautiously treat it with 10 per cent. acetic acid from a burette, noting the original level of the acid in the burette. Add the acid a drop or two at a time, shaking gently the whole time, until the precipitate produced at each addition no longer dissolves on shaking, and the whole mixture is rather opalescent. This point is usually somewhat difficult to determine owing to the large number of air-bubbles that become suspended in the fluid and closely resemble a fine precipitate. When you are satisfied that a permanent precipitate has been produced run in 1 c.c. of the acid in addition to the amount already added, a heavy white precipitate is thus produced. Note the amount of acid that has been used for the portion of 100 c.c., and treat the remainder of the filtrate with a corresponding amount of acid. Mix the two portions thoroughly and allow it to stand overnight. Note that the precipitate has increased somewhat in amount. Mount a drop of the suspension on a slide, cover with a slip, but do not press. Examine under the high power of the microscope, and note the aggregation of very fine needles.

The albumin can be recrystallised by filtering, dissolving in as small an amount of water as possible, filtering again, and cautiously adding to the filtrate saturated ammonium sulphate till a faint permanent precipitate is produced. If the mixture be allowed

to stand for some hours the albumin will separate out as fine needles.

NOTES—1. For the experiment to succeed it is absolutely essential that all the eggs employed be perfectly fresh. One rather stale egg may interfere with the crystallisation of a large number of fresh eggs.

2. It is important to add exactly the amount of acetic acid mentioned, that is, one per mille above the amount required to give a faint permanent precipitate.

3. The same method can be employed for the crystallisation of serum-albumin from the perfectly fresh serum of a horse, ass or mule.

D. The Meta-proteins.

25. Egg-white or serum has been diluted with ten times its volume of either ·4 per cent HCl or ·1 per cent. Na OH and the mixture placed in a water bath at 40°C for about twenty-four hours. The proteins have thus been converted to meta-proteins.

To about twenty-five c.c. add a few drops of litmus and carefully neutralise with 2 p.c. Na_2CO_3 or ·4 p.c. HCl. A bulky precipitate of meta-protein separates out. Filter. Scrape the precipitate off the paper and suspend it in a test-tube about half-full of water. Divide the suspension into six equal portions and with them perform the following six exercises.

26. Add some 4 p.c. HCl. The precipitate dissolves. Neutralise with Na_2CO_3: the precipitate reappears.

27. Add concentrated HCl drop by drop. The precipitate dissolves with the first drop, and reappears when an excess is added.

28. Dissolve in a little ·4 p.c. HCl. Boil the solution: a coagulum is not formed. Cool under the tap and neutralise with 2 p.c. Na_2CO_3. A precipitate is formed which is soluble in an excess.

29. Boil. Cool and add some ·4 p.c. HCl. The precipitate does not dissolve, *i.e.*, meta-protein is coagulated when boiled in suspension.

30. Add a saturated solution of ammonium sulphate drop by drop. The precipitate does not dissolve in any dilution of the salt.

31. Dissolve in a little ·4 p.c. HCl. Treat the solution with an equal volume of saturated ammonium sulphate solution. The protein is precipitated.

NOTES—1. The meta-proteins are formed rapidly by the action of dilute acids or alkalies on albumins or globulins at temperatures above 60°C. (See Notes to Ex. 8); more slowly at body-temperature.

2. They are insoluble in water, *strong* mineral acids, and all solutions of neutral salts, but are soluble in dilute acids or alkalies in the absence of any large amount of neutral salts.

3. They are not thrown out of solution by boiling.

4. If they are precipitated by neutralisation and then boiled they are coagulated, so that they are no longer soluble in dilute acids or alkalies.

5. The substance formed by the action of acids on albumin or globulin does not differ in its properties from that formed by the action of alkalies on albumin or globulin.

E. Myosin.

32. Fresh veal is finely minced in a machine, stirred with a large volume of water for a quarter of an hour,

strained through muslin, and the washing process repeated once more. In this way certain proteins and other substances soluble in water are removed. The veal is now collected on muslin, squeezed to remove the water, ground with sand, and extracted with five times its volume of 10 per cent. ammonium chloride for several hours at room temperature. The extract is filtered through muslin, linen, and then coarse filter paper. In this way a crude, viscid solution of myosin is obtained.

33. Boil a portion of the solution. A heavy coagulum is formed. Wash the coagulum and on it perform the protein colour reactions. They are all obtained.

34. Pour 100 c.c. into a litre of water contained in a tall cylinder; mix well, and note the precipitation of myosin. Allow this to settle and then pour or pipette off as much of the supernatant fluid as possible. A suspension of myosin in dilute ammonium chloride is thus obtained for the following experiments:

35. To a portion add a saturated solution of common salt, drop by drop. The precipitate dissolves. Add solid NaCl to saturation: the myosin is reprecipitated.

36. To a portion add saturated $(NH_4)_2SO_4$ till the precipitate just dissolves. Now add an equal bulk of saturated $(NH_4)_2SO_4$. The myosin is reprecipitated.

37. Dissolve in a little $(NH_4)_2SO_4$ and take the temperature at which the myosin coagulates. It coagulates at about 57°C. (See Ex. 9.)

NOTE.—It will be seen from the above reactions that myosin is a member of the globulin class of proteins, *i.e.*, it coagulates on boiling, is insoluble in distilled water; soluble in dilute salt solutions; precipitated by half-saturation with $(NH_4)_2SO_4$ or by saturation with NaCl.

F. Mucin.

Preparation. Mince the submaxillary gland of an ox, grind with sand and add ·1 per cent. NaHO (1 litre to 50 grams. of the moist gland). Shake well in a large bottle from time to time and leave for about half an hour. Strain through muslin and filter through coarse filter paper. (This crude solution should not be prepared too long before use, as mucin loses its characteristic properties if left standing with alkalies.)

38. Add acetic acid drop by drop. A stringy precipitate is formed, insoluble in excess of the acid.

39. Remove the precipitate on a glass rod, wash with water, and apply the usual colour reactions for proteins, *e.g.* xanthoproteic, glyoxylic, and Millon's. They are all given by mucin.

40. Treat some of the precipitate with ·1 per cent. HCl. The mucin dissolves.

41. Treat some of the precipitate with 2 per cent. Na_2CO_3. The mucin dissolves.

G. Nucleo-protein and Nucleo-histone.

Preparation. Lymphatic glands of the ox or sheep, or the thymus of a calf are freed from fat, finely minced, ground with sand and extracted for twelve hours with ten times their weight of distilled water in a large bottle, a small amount of toluol or chloroform being added to prevent decomposition. The bottle should be frequently shaken vigorously to break up the gelatinous masses that sometimes form. The fluid is strained and centrifugalised to remove all débris (filtration being very slow). This fluid contains both nucleo-protein and nucleo-histone.

42. To a portion add dilute acetic acid till no more precipitate is produced, and place on the water-bath at 37°C. for a few minutes. A heavy precipitate of nucleo-protein and nucleo-histone is formed. Allow this to settle in a cylinder: pour or pipette off as much of the supernatant fluid as possible, and filter the remainder. Note that the precipitate is soluble in dilute alkalies and is reprecipitated by acidification; that it dissolves to an opalescent solution in excess of acetic acid (difference from mucin); and that it gives all the usual colour reactions for proteins.

43. To another portion add one-tenth of its volume of 2 per cent. calcium chloride and warm to 37°C. A white precipitate of nucleo-histone is formed. Pour off the supernatant fluid, and to this fluid add dilute acetic acid drop by drop; a white precipitate of nucleo-protein is produced.

44. Precipitate the nucleo-protein and nucleo-histone from the remainder of the fluid by means of acetic acid as in Ex. 42. Collect the precipitate on a filter paper, allow it to drain well, and then transfer it by means of a spatula to a small thimble-shaped porcelain capsule. Heat carefully to drive off the water and then to carbonise the residue. Add one-third of a crucible full of fusion mixture (K_2CO_3 two parts, KNO_3 one part), and heat as strongly as possible till the mass fuses. Allow the melt to cool, and extract it with nitric acid diluted with an equal quantity of distilled water till the mixture no longer effervesces. Filter: treat the filtrate with about one-tenth its volume of strong nitric acid and one-third its volume of ammonium molybdate: boil for two minutes. The yellow crystalline precipitate separating out on the sides of the tube shows that nucleo-proteins and nucleo-histone contain phosphorus, that has been oxidised to a phosphate by the fusing process.

H. The Albumoses and Peptones.

For the preparation of albumoses and peptones, see Ex. 148. For the following reactions make a 5 per cent. solution of "Witte's peptone" in hot water, and filter from a small amount of insoluble material. The solution contains all the albumoses and peptones.

45. Dilute a small amount with three or four times its bulk of water, and to portions of this apply the usual colour reactions for protein. They are all

obtained. Note, in particular, that the biuret test gives a rose colour.

46. Boil the solution with a trace of acetic acid: it does not form a coagulum.

47. Add a little tannic acid: a white precipitate is formed.

48. Add a little lead acetate solution: a white precipitate is formed.

49. To 10 c.c. of the 5 per cent. solution in a small beaker add 10 c.c. of a saturated solution of ammonium sulphate. A white precipitate of the primary albumoses is formed. Stir the mixture vigorously for a short time with a glass rod that has one end covered with a small piece of rubber tubing: allow to stand for a few minutes. The precipitate will usually gather together and can be almost completely collected as a gummy mass on the end of the rod. Transfer it to about 5 c.c. of hot water. The precipitate dissolves. Cool the solution and divide it into three portions.

(*a*) Add a drop of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed, which disappears on heating and reappears on cooling.

(*b*) To another portion add a few drops of strong nitric acid. A white precipitate is formed, which disappears on heating and reappears on cooling.

(*c*) To the third portion add a drop of copper sulphate solution. A white precipitate is formed.

50. The fluid from which the main mass of primary albumoses has been removed is filtered and treated in a beaker with a single drop of sulphuric acid, and then with ammonium sulphate that has been finely powdered in a mortar. The mixture is stirred vigorously till the fluid is saturated with the salt. A flocculent precipitate of the secondary albumoses (deutero-albumoses) is formed. Collect this on the rod as before, dissolve in a little water, divide the solution into three parts, and repeat the three tests already performed with the primary albumoses. A precipitate is not formed by any of the reagents.

51. The fluid from which the secondary albumoses have been removed contains peptone. Filter it, and treat a portion of the filtrate with twice its volume of 40 per cent. sodium hydrate and a drop of 1 per cent. copper sulphate. A pink colour appears, due to the presence of peptone.

Important Note.—This large excess of strong NaOH must be added in order to decompose the $(NH_4)_2SO_4$ with which the solution is saturated. The characteristic rose colour is only obtained when the alkalinity is due to NaOH, ammonia being quite inefficient.

52. Evaporate a small portion of the original fluid to complete dryness, finishing the process on a water bath in order to prevent charring. Rub up the residue with successive small quantities of strong alcohol (95 per cent.). Add the extracts together, filter and evaporate them to dryness on a water bath. Dissolve the residue from this evaporation in a little

water and test for proteins by the various colour tests. Only insignificant traces are present, showing that albumoses and peptones are insoluble in strong alcohol.

NOTE.—It is frequently desirable to remove all proteins from a solution before testing for certain substances, *e.g.*, sugars, bile-salts, urea, etc. In the case of albumoses and peptones this can only be effected by the method described above, advantage being taken of the solubility of sugars, etc., in alcohol, and the insolubility of all proteins in the same. The aqueous solution prepared in this way will be spoken of as "an alcoholic extract."

Peptones. Use a 2 per cent. solution of Savory and Moore's peptone, which is usually free from albumoses.

53. Apply the usual colour reactions for proteins. They are all obtained.

NOTE.—The glyoxylic reaction may not be very intense, owing to the presence of chlorides in the preparation. Pure peptone, when freed from chloride by appropriate means, gives a very good glyoxylic reaction.

54. Add a drop or two of strong acetic acid and a drop of potassium ferrocyanide. No precipitate is produced, showing that the primary albumoses are absent.

55. Saturate a portion with $(NH_4)_2SO_4$. No precipitate is produced, showing that all albumoses are absent.

56. Treat the filtrate from (Ex. 55) with two volumes 40 per cent. NaOH and a drop of copper sulphate. A rose colour is obtained, showing that peptone is present.

57. Add a few drops of a solution of tannic acid. A white precipitate is formed.

58. Add a few drops of a solution of lead acetate. A white precipitate is formed.

NOTES—1. There are five albumoses obtained by the action of pepsin and dilute hydrochloric acid on fibrin.

The **primary albumoses** are precipitated by half saturation with $(NH_4)_2SO_4$. They give a precipitate that dissolves on warming and reappears on cooling either with nitric acid or with potassium ferrocyanide and acetic acid; they also give a precipitate with copper sulphate. There are two primary albumoses, *viz.* hetero- and proto-albumose, the latter being distinguished by its solubility in 60 per cent. alchol.

There are three **deutero-albumoses** (secondary albumoses).

I. is precipitated by two-thirds saturation with $(NH_4)_2SO_4$
II. „ „ complete „ „ „
III. „ „ „ „ „ „
in the presence of a little sulphuric acid.

They do not give any of the three characteristic reactions of the primary albumoses.

2. Peptone is the name given to the protein formed by the prolonged action of pepsin and hydrochloric acid on fibrin, egg-white, etc.

Peptone is not precipitated by saturation with any mixture of salts, and is thus distinguished from the albumoses and all other proteins.

3. In testing for albumoses and peptones the fluid must first be freed from acid or alkali albumin by neutralisation, and from albumin and globulin by boiling. The filtrate is then saturated with $(NH_4)_2SO_4$: the precipitate will contain the albumoses, identified by the usual protein tests, whilst the filtrate will contain the peptone, which is identified by the biuret test as described in Ex. 51.

I. The Reactions of certain Sclero-proteins.

59. **Gelatin.** Break gelatin up into small pieces and add a small amount of cold water. The gelatin does not dissolve. Immerse the test tube in a beaker of boiling water and leave it for a short time. The gelatin dissolves. Cool the tube under the tap: the gelatin sets to a jelly.

Perform the following tests with about a 1 per cent. solution of gelatin.

(*a*) Xanthoproteic reaction: slight.

(*b*) Millon's reaction: very slight, showing absence of tyrosin from gelatin molecule. (See Notes to Ex. 2.)

(*c*) Glyoxylic reaction: not obtained, showing absence of tryptophane. (Ex. 3.)

(*d*) Biuret reaction: violet colour.

(*e*) Sulphur reaction: not obtained, showing absence of cystin (Ex. 5).

(*f*) Add acetic acid: no precipitate.

(*g*) Add acetic acid and potassium ferrocyanide: very slight or no precipitate.

(*h*) Add tannic acid: white precipitate.

(*i*) Add lead acetate: very slight or no precipitate.

(*k*) Saturate with ammonium sulphate. The whole of the gelatin is precipitated, as shown by a negative biuret test in the filtrate (distinction from peptones).

60. **Keratin.**

Perform the following tests by using horn shavings, or hair. Note insolubility in hot or cold water, dilute acids, and dilute alkalies.

(*a*) Xanthoproteic reaction: well marked.

(*b*) Millon's reaction: well marked.

(*c*) Glyoxylic reaction: well marked.

(*d*) Biuret reaction: not obtained, owing to insolubility.

(*e*) Sulphur reaction: well marked.

CHAPTER II.

THE CARBOHYDRATES.

A. The Mono-saccharides.

Dextrose (glucose or grape-sugar).

Use a ·2 per cent. solution for the following reactions.

61. Boil with a little sodium hydrate. The solution turns yellow. (**Moore's test.**)

NOTE.—The yellow colour is due to the formation of caramel (a condensation product) by the hot alkali. It must be noted here that dextrose is completely destroyed by prolonged boiling with alkali.

62. Treat two or three c.c. of 5 per cent. caustic soda with four or five drops of a 1 per cent. solution of copper sulphate. A blue precipitate of cupric hydrate, $Cu(OH)_2$, is formed. Add to the mixture an equal bulk of the sugar solution. The precipitate dissolves. Boil the solution for a short time. The blue colour disappears, and is replaced by a yellow or red precipitate of cuprous oxide, Cu_2O. (**Trommer's test.**)

NOTES—1. The amount of copper necessary depends on the percentage of sugar present. If only a small amount of sugar be present a mere disappearance of the blue colour is all that

may happen, or possibly the fluid may assume a faint yellowish-red tint. If excess of copper be added, the reduction is obscured by the blue cupric hydrate in solution, or the black precipitate of cupric oxide that is formed on heating this in the alkaline solution. It is always best to add the copper sulphate a few drops at a time, boiling between each addition.

2. The reaction is a type of several that have been introduced for the detection of dextrose, all of which depend on the fact that in alkaline solution it has reducing properties when boiled. For this reason, dextrose, and all sugars that have this property are sometimes spoken of as "reducing sugars."

3. The property that dextrose and other sugars have of dissolving cupric hydrate is common to a large number of organic compounds.

63. Boil about 3 c.c. of Fehling's solution (See Note 1) in a test tube. No change occurs. Add about 3 c.c. of the dextrose solution and boil again. A red precipitate of cuprous oxide is formed (**Fehling's test.**)

NOTES—1. Fehling's fluid is prepared as follows:

(*a*) Dissolve 103·92 grams. of pure copper sulphate in warm water and dilute to one litre.

(*b*) Dissolve 320 grams. of potassium sodium tartrate (Rochelle salt) in warm water, add a little carbolic acid to prevent the growth of fungi, dilute to exactly a litre and filter.

(*c*) Dissolve 150 grams. of sodium hydrate in distilled water and dilute to 1 litre.

For use take exactly equal quantities of *a*, *b* and *c*, and mix. Though the individual constituents keep indefinitely, the fluid when prepared suffers decomposition, so that a reduction occurs on boiling. For this reason the fluid should be prepared just before use, and must always be tested by boiling before being used.

The fluid is of such a strength that the copper sulphate in 10 c.c. is just reduced by ·05 grams. of dextrose.

2. The addition of the Rochelle salt is for the purpose of dissolving the cupric hydrate that would otherwise be precipitated by mixing (*a*) and (*c*).

3. The test is much more delicate and certain than Trommer's test, and should always be used in preference to it.

4. If the fluid that is being tested is acid, it should be neutralised.

5. Ammonium salts considerably interfere with Fehling's test. If they are present a little extra alkali should be added, and the mixture boiled for two or three minutes to allow of the evolution of the ammonia.

64. Treat 2 c.c. of a ·1 per cent. solution of safranine with 2 c.c. of the dextrose solution and 2 c.c. of 5 per cent. sodium hydrate. Mix and boil, avoiding any shaking. The opaque red colour gives place to a light yellow, owing to the reduction of the safranine to a "leuco-base."

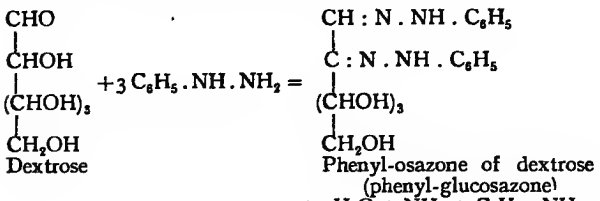
65. Add to the solution of dextrose some solution of sulphindigotate of soda and some Na_2CO_3 and boil. The blue colour turns green, purplish, red, and finally yellow. Shake with air: the blue colour reappears. **(Mulder's test.)**

NOTE.—These two experiments illustrate the reducing properties of dextrose in hot alkaline solution. The avidity of the reduced leuco-bases for oxygen is shown by the reappearance of the colour on cooling and shaking with air.

66. Take 10 c.c. of a 1·5 per cent. solution of dextrose in a test tube. Add as much solid phenylhydrazine-hydrochloride as will lie on a sixpenny piece, and at least twice this amount of solid sodium acetate. Dissolve by warming, mix thoroughly, and filter into a clean test tube. Place this in a beaker of boiling water for at least half-an-hour, keeping the water boiling the whole time. Set the tube aside to cool (do not cool under the tap). A fine yellow

crystalline precipitate of phenyl-glucosazone appears. Collect some of this by means of a pipette, transfer to a slide, cover with a glass and examine under both powers of the microscope. Note the characteristic arrangement of the fine yellow needles in fan-shaped aggregates, sheaves or crosses. Make a drawing of the crystals in the space provided at the end of the book.

NOTES—1. Dextrose is an aldehyde, and, like all aldehydes and ketones, forms a compound with phenyl-hydrazine. But this pheynl-hydrazone of glucose is very soluble, and cannot be readily separated. However, in the presence of an excess of phenyl-hydrazine at 100°C. an insoluble osazone is formed.



2. Phenyl-hydrazine is a yellow fluid-base, insoluble in water, but soluble in dilute acids to form salts. If the base itself is used, two or three drops should be dissolved in a few drops of strong acetic acid, and added to the sugar solution.

3. Phenyl-hydrazine-hydrochloride, $C_6H_5 . NH . NH_2 . HCl$ does not give an osazone when boiled with dextrose, unless an excess of sodium acetate be added. This acts on the hydrochloride to form phenyl-hydrazine-acetate and sodium chloride.

4. All the "reducing-sugars" form osazones when treated in the way described above; but the osazones differ in crystalline form, and especially in their solubility in hot water. Laevulose (fruit-sugar), however, forms the same osazone as does dextrose.

5. Glucosazone melts at 205°C.

B. The Disaccharides.

Lactose (Milk-sugar, $C_{12}H_{22}O_{11}$).

67. Repeat experiments 61, 62, and 63 with a ·2 per cent. solution of lactose. It reacts exactly like dextrose.

68. Examine microscopically and draw the crystals of phenyl-lactosazone that have been prepared by the demonstrator. Notice that they differ considerably from glucosazone, separating, usually, as ovoid or spherical clusters of fine needles. Make a drawing of the crystals in the space provided at the end of the book.

NOTES—1. Lactosazone melts at 200°C, and has a compositon, $C_{24}H_{32}N_4O_9$. It is soluble in 80 to 90 parts of boiling water.

2. Lactose is converted to equal parts of dextrose and galactose by boiling with dilute mineral acids, or by treatment with the ferment lactase at 37°C.

$$\underset{\text{Lactose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose}}{C_6H_{12}O_6} + \underset{\text{Galactose.}}{C_6H_{12}O_6}$$

3. The reducing power of lactose towards Fehling's fluid is 52·5 per cent. of that of dextrose.

4. Lactosazone prepared directly from urine containing lactose usually separates out as crescentic masses.

Maltose ($C_{12}H_{22}O_{11}$).

69. Repeat experiments 61, 62, and 63 with a ·2 per cent. solution of maltose. It reacts exactly like dextrose.

70. Examine microscopically and draw the crystals of phenyl-maltosazone that have been prepared by the demonstrator. Note that they are much broader

than the crystals of glucosazone. Make a drawing of the crystals in the space provided at the end of the book.

NOTES—1. Maltosazone melts at 206°C., and has a composition $C_{24}H_{32}N_4O_9$. It is soluble in 75 parts of boiling water.

2. Maltose is converted to dextrose by boiling with dilute mineral acids, or by treatment with the ferment maltase at 37°C.

$$\underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + H_2O = 2\underset{\text{Dextrose}}{C_6H_{12}O_6}$$

3. The reducing power of maltose towards Fehling's fluid is 61 per cent. of that of dextrose, *i.e.* 100 grams. of maltose reduce the same amount of Fehling's fluid as does 61 grams. of dextrose.

Cane-sugar (Saccharose, $C_{12}H_{22}O_{11}$).

71. Repeat experiments 61, 62, and 63 with a freshly-prepared 1 per cent. solution of pure white crystalline cane-sugar. Note that it does not give these reducing reactions.

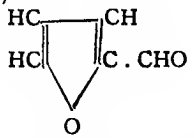
72. Treat 3 c.c. of the solution with one drop of strong sulphuric acid and boil for a minute. Add a drop of litmus solution and neutralise with caustic soda. Apply Trommer's or Fehling's test to portions of this fluid. A well marked reduction is obtained in both cases.

NOTE.—This reaction depends on the fact that although cane-sugar is a non-reducing sugar, it is converted to equal parts of dextrose and laevulose by boiling with dilute mineral acids.

$$\underset{\text{Cane-sugar.}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose.}}{C_6H_{12}O_6} + \underset{\text{Laevulose.}}{C_6H_{12}O_6}$$

73. Treat four drops of the solution of cane-sugar with four drops of 2 per cent. solution of alpha-naphthol in alcohol and 5 c.c. of fuming hydrochloric acid. Boil for a minute. The fluid turns a rich purple colour.

NOTES—1. This reaction depends on the fact that the laevulose which is formed by the action of the acid on the cane-sugar yields furfurol (furfuraldehyde)



which in its turn reacts with the alpha-naphthol to give a purple colour.

2. Dextrose, lactose, and maltose only give this reaction very feebly. The polysaccharides and especially cellulose give a fair reaction. It is also given by certain proteins, when it is known as Molisch's reaction.

3. In using the reaction as a test for cane-sugar, great care must be taken to remove proteins and dextrins from solution by the method described in Ex. 52. The residue left after evaporation of the alcohol will contain all the sugars present in the original fluid.

4. Thymol can be used instead of alpha-naphthol.

74. Mix a solution of cane-sugar with one of dextrose. Boil the mixture with Fehling's solution, adding the Fehling's solution to the boiling fluid until a blue colour by transmitted light indicates a slight excess of Fehling's solution. By this procedure the dextrose is destroyed, but the cane-sugar is unaffected. Filter off the precipitate of cuprous oxide. Make the filtrate acid with sulphuric and boil. Neutralise the solution, add a little more Fehling's and boil again. A well-marked reduction is obtained due to the production of dextrose and laevulose by "inversion" of the cane-sugar by the acid.

NOTE.—In using this as a test for cane-sugar in the presence of dextrose, the presence of the polysaccharides must be excluded by alcoholic extraction if necessary: and the solution must give a well-marked alpha-naphthol test, as lactose and maltose, after boiling with Fehling's solution, give a reducing substance by acid hydrolysis.

C. The Polysaccharides.

Starch.

75. Place a small amount of dry potato-starch on a slide, add a drop of water, cover with a slip and examine under the microscope. Note the characteristic oval starch grains, the concentric markings and the hilum, usually eccentric. Make a drawing of the grains. Run a drop of iodine under the slip; note that the grains take on a blue colour.

76. Shake a small amount of potato starch with cold water. The starch does not dissolve. Filter, and add a drop of iodine solution to the filtrate. The characteristic blue reaction is not obtained.

77. Shake some dry starch with a little sodium carbonate. No change is effected. Repeat, with a little sodium hydrate. The starch is immediately gelatinised. Add a few drops of iodine solution, a blue colour is not obtained. (See note to Ex. 78).

78. Take as much starch as will lie on a shilling, shake it up with 5 c.c. of water, and pour into 100 c.c. of boiling water, stirring the mixture during the addition. Boil for two minutes. The starch becomes gelatinised, and forms a thin, somewhat opalescent paste. Cool a portion under the tap and add a drop of iodine solution. A deep blue colour is formed.

NOTE.—In testing for the polysaccharides by iodine, the reaction of the fluid must not be alkaline. Alkalies convert iodine to iodides and iodates, which do not react with these carbohydrates.

79. Treat 5 c.c. of the cold starch paste with an equal bulk of saturated ammonium sulphate. Shake the test tube and allow it to stand for five minutes. The starch is precipitated. Filter through a dry paper, and add a drop of iodine solution to the filtrate. No blue colour, or only the very slightest tint is obtained, showing that the whole of the starch paste is precipitated by half-saturation with $(NH_4)_2SO_4$.

80. Boil 5 c.c. of the starch paste with two drops of concentrated sulphuric acid for about 15 seconds. Note that the solution becomes perfectly clear and translucent. Add two drops of strong ammonia to neutralise the acid, cool under the tap, add an exactly equal bulk of saturated $(NH_4)_2SO_4$, shake the tube vigorously and allow it to stand for five minutes. Filter through a dry filter-paper and add two drops of iodine solution to the filtrate. A deep blue colour is obtained.

NOTE.—Starch paste is rapidly converted to "soluble starch" on boiling with dilute mineral acids. Soluble starch differs from starch paste in that it is not completely precipitated by half-saturation with $(NH_4)_2SO_4$ in the course of five minutes. If it be allowed to stand for twenty-four hours, however, it is completely precipitated.

81. Take 10 c.c. of the starch paste in a small beaker. Add five drops of concentrated sulphuric acid, bring the mixture to the boiling point, and keep it boiling for five minutes. Add a drop of litmus solution and neutralise with sodium hydrate, keeping the reaction on the acid rather than the alkaline side. Cool one portion under the tap and add a drop of

iodine solution. A purple, red or brown reaction of erythro-dextrin is obtained, instead of the original blue reaction of starch. To the other portion add 3 c.c. of Fehling's solution and boil. A well marked reduction is obtained.

NOTE.—Starch is converted to erythro-dextrin and dextrose by boiling with dilute mineral acids. If the boiling is prolonged the erythro-dextrin is converted to dextrose. The extent of boiling required to destroy the whole of the starch, and yet to leave some erythro-dextrin varies with the concentrations of the starch paste and of the acid employed.

The Dextrins.

82. Shake a little commercial dextrin with some cold water. An opalescent solution is formed. Boil the solution. It becomes perfectly translucent. (Distinction from glycogen.)

Use a 3 per cent. solution of commercial dextrin for the following reactions.

83. To about 5 c.c. of the dextrin solution add iodine solution, drop by drop, noting the colour at every addition. The colour is at first almost a pure blue but it changes through a rich purple-red to a red-brown as the iodine is added.

84. Repeat the above experiment, but boil and then cool the tube after each addition. The colour disappears on boiling, but does not reappear on cooling until several drops of iodine have been added.

85. Add a drop or two of the starch paste prepared in Ex. 78 to about 5 c.c. of the dextrin solution. To this mixture add diluted iodine solution, drop by drop.

The first additions produce a pure blue colour, and it is not till a considerable amount of iodine has been added that the solution acquires a purplish tint.

NOTE.—The affinity of starch for iodine is so much greater than that of dextrin that the characteristic colour reactions of erythro-dextrin are not obtained until all the starch has been saturated with iodine. Even then it is sometimes difficult to detect, owing to the deep blue starch reaction.

86. Treat 5 c.c. of the dextrin solution with about 10 drops of the starch paste: to the mixture add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. The purple-red reaction of erythro-dextrin is obtained.

87. Saturate 5 c.c. of the dextrin solution with finely powdered $(NH_4)_2SO_4$. Note the precipitate of erythro-dextrin produced. To the filtrate add a drop of iodine. A red-brown colour is produced. Scrape the precipitate off the paper, dissolve it in a little hot water, cool, and add iodine. A purple-red solution is obtained.

NOTE.—There are two chief classes of dextrins known. The erythro-dextrins give a colour reaction with iodine, whilst the achroo-dextrins give no colour with iodine.

There are three closely related erythro-dextrins, which are usually designated I., II., and III. respectively. Of these I. and II. are precipitated by saturation with $(NH_4)_2SO_4$, whilst III. is not. I. differs from II. in being precipitated by saturation with $MgSO_4$. The colour reactions with iodine also differ, I. giving a purple, II. a red, and III. a red-brown.

88. Boil a few c.c. of the dextrin solution with a small amount of Fehling's fluid. A well-marked reduction is usually obtained.

NOTE.—Commercial dextrin is generally prepared by the action of dilute acids on starch (See Exercises 80 and 81), the action being stopped as soon as a portion fails to give a blue colour with iodine, and the products then being precipitated by alcohol. Such preparations contain some dextrose, and often a little soluble starch. At the same time it must be noted that the achroo-dextrins have a reducing action themselves even when thoroughly separated from the dextrose.

89. Take 10 c.c. of the dextrin solution in a small flask; add 30 c.c. of strong (95 per cent.) alcohol, place the thumb over the mouth of the flask and shake vigorously for some seconds. Note that a portion of the dextrin is precipitated as a gummy mass which sticks to the sides of the flask.

Pour off the alcohol, filter it and label the filtrate A. Rinse the flask out with a few c.c. of alcohol, shake off as much of this alcohol as possible, and add 10 c.c. of hot water. Shake this round the flask till the whole of the gummy precipitate dissolves. Divide the solution into three portions, B, C, and D. To B add a drop of iodine: a purple colour is produced. Boil C with a little Fehling's solution: no reduction takes place. Boil D with two drops of concentrated sulphuric acid for two minutes, neutralise with NaOH, and boil with a little Fehling's solution: a well-marked reduction occurs.

90. To a portion of filtrate A, add a drop of iodine solution. No colour is produced. To another portion

of about 5 c.c. add an equal bulk of strong alcohol. A white precipitate of achroo-dextrin is formed.

NOTE.—We learn from these two last exercises that erythro-dextrin is completely precipitated by 71 per cent. alcohol: that it does not reduce Fehling's solution: that it is converted to dextrose by boiling with mineral acids; and that achroo-dextrin is more soluble in alcohol than erythro-dextrin.

Glycogen.

91. *Preparation.*—A rabbit, which has had a full meal of carrots some five or six hours previously, is killed by decapitation. The liver is cut out as quickly as possible, and the gall-bladder removed. The liver is rapidly chopped into small pieces, a small portion is reserved for Exercise 97, and the remainder immediately thrown into boiling water. After about two minutes boiling the larger morsels are strained off, pounded to a paste with sand in a mortar, and replaced in the boiling water. The proteins in solution are then coagulated by making the boiling fluid just acid with acetic acid. The fluid is filtered through coarse filter paper. In this way a crude solution of glycogen is obtained.

92. Boil 5 c.c. in a test tube. The characteristic opalescence does not disappear. (Distinction from erythro-dextrin.)

93. To a small amount of the cooled solution add iodine, drop by drop. A red colour is formed, which disappears on shaking, until with a certain amount of iodine added it is permanent. Now heat the solution. The colour disappears, to reappear on cooling.

NOTE.—If much protein is present in solution the colour will not reappear on cooling unless a considerable amount of iodine be added. This is due to the fact that proteins combine with iodine to form an iodo-protein.

94. Saturate 10 c.c. of the solution with finely-powdered $(NH_4)_2SO_4$. The glycogen is precipitated. Filter, and add a drop or two of iodine to the filtrate.

No red colour is produced. Scrape the precipitate off the paper, boil with a small amount of water. The solution is markedly opalescent. Cool the solution, and add iodine. A port-wine red colour is obtained.

95. Boil 5 c.c. of the solution with a little Fehling's fluid. A very slight or no reduction is obtained.

NOTE.—If the liver has been rapidly boiled, no sugar will be present. If delay has occurred during the initial stages of the preparation, some of the glycogen will have been converted to dextrose (See Exercise 97).

96. To 10 c.c. of the solution add 20 c.c. of strong alcohol, shake vigorously and filter. To a portion of the filtrate add iodine solution. No colour is obtained, showing that the whole of the glycogen is precipitated. Dissolve the precipitate in a little hot water: note that it is opalescent. Add three drops of strong sulphuric acid and boil for about three minutes: the opalescence disappears. Neutralise with sodium hydrate and apply Fehling's test. A marked reduction occurs, due to the conversion of the glycogen into dextrose by the boiling acid.

97. The portion of rabbit's liver that was reserved in Exercise 91, has been kept in a warm place for about six hours and extracted with boiling water as in Exercise 91. (Or a decoction of the liver of a sheep obtained from a butcher may be used.) Note that the solution is almost translucent. To a portion add iodine: only a very slight or no red colour at all is produced. To another portion apply Fehling's test: a well-marked reduction occurs.

NOTES—1. Glycogen differs from erythro-dextrin in being markedly opalescent in solution; in being completely precipitated by saturation with $(NH_4)_2SO_4$, and in being more readily precipitated by alcohol. Like erythro-dextrin it is converted to dextrose by boiling with dilute mineral acids. It has no reducing properties.

2. Exercise 97 illustrates the property that the liver has of hydrolysing glycogen to dextrose, the action being due to a ferment that is destroyed by boiling.

98. Prepare a solution which contains equal quantities of 1 per cent. starch paste (freshly prepared), of a strong solution of glycogen and of a 3 per cent. solution of commercial dextrin. Note that the mixture is markedly opalescent.

To a small portion add diluted iodine, and note that a pure blue *starch* reaction is obtained.

To another portion of about 5 c.c. add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, leave for five minutes, and filter. Note that a portion of the filtrate gives a reddish colour with iodine, and that it is distinctly opalescent. Indication of the presence of *glycogen.*

Saturate the remainder of the fluid with finely-powdered $(NH_4)_2SO_4$ and filter. The filtrate gives a reddish-brown colour with iodine. Indication of the presence of *erythro-dextrin.*

D. The Quantitative Estimation of Dextrose.

The basis of nearly all the modern methods for the volumetric estimation of the sugars is the determination of the amount of the sugar solution necessary to reduce a given volume of Fehling's solution. The chief difficulty of the method lies in deciding the

exact point when the copper is reduced, as indicated by the complete disappearance of the blue colour. This is obscured by the red precipitate of cuprous oxide that is formed. To obviate this difficulty Pavy introduced the ammoniacal process that bears his name. The ammonia keeps the cuprous oxide in solution as a colourless compound, so that the disappearance of the blue colour is readily observed. The objection to the method is that the ammonia is evolved as the solution boils and considerable experience is required to know the proper rate at which to add the sugar solution. Though, in the hands of the expert the results obtained by Pavy's method are extremely accurate, in class work it is not very satisfactory and I have now discontinued using it for such purposes.

I have not been able to decide as to which is the better of the two new methods given below. But owing to Ling's method being the simpler it will probably prove the more convenient for general work.

99. **Ling's method for the estimation of dextrose.**

Principle of the Method. A measured volume of Fehling's solution is boiled and whilst boiling is titrated with the dextrose solution until the cupric salt is converted to cuprous. The end-point is determined by means of an acid solution of ferrous thiocyanate as an indicator. If this be treated with a cupric salt, the colourless ferrous thiocyanate is oxidised to the red ferric thiocyanate.

Reagents required. (i.) Fehling's solution, freshly prepared according to the directions given in Ex. 63.

(ii.) The indicator, consisting of

Ammonium thiocyanate	1·5 grams.
Ferrous ammonium sulphate	1·0 gram.
Concentrated hydrochloric acid	2·5 c.c.
Water...	10 c.c.

The thiocyanate and the ferrous sulphate are dissolved in 10 c.c. of water at about 45°C and immediately cooled. The hydrochloric acid is then added. The solution so obtained has invariably a brownish-red colour, due to the presence of ferric salt, which latter must therefore be reduced by the addition of a trace of zinc dust. The indicator when kept for some hours develops the red colour by atmospheric oxidation. It may, however, be decolourized by the addition of a further quantity of zinc dust, but its delicacy is impaired after it has been decolourized several times.

Method of Analysis. Fill a burette with the sugar solution. Accurately measure 10 c.c. of Fehling's solution into a flask and boil it. Slowly run the sugar into the Fehling's solution until the blue colour has nearly gone. To prevent, as far as possible, the oxidation of the cuprous oxide by the air **the mixture must be kept boiling the whole time.**

Place a few drops of the indicator on a clean porcelain or opal glass slab. Withdraw a drop of the mixture from the boiling flask and transfer it to one of the drops on the slab by means of a glass rod or capillary tube. The drop must be transferred as rapidly as possible, to prevent the oxidation of the cuprous compound by the air. If a red colour is produced, add some more sugar to the flask, wait for about 15 seconds and transfer another drop to the slab. Repeat until no red colour is produced on mixing. Read off the amount of sugar solution added. If it is less than 20 c.c., accurately dilute the solution until between 20 and 30 c.c. are required for 10 c.c. of Fehling's solution. Repeat the process with the diluted solution.

Calculation of Results.

A near approximation can be obtained on the basis that 10 c.c. of Fehling's solution are reduced by ·05 gram. dextrose, and such a method serves for all ordinary work.

It would be more accurate to standardise the Fehling's solution under the conditions of the exact method employed by each individual by the use of a standard solution of invert sugar prepared as follows:— 9·5 grams. of pure cane sugar are dissolved in water and the solution accurately made up to 1000 c.c. Of this solution 100 c.c. are boiled with 30 c.c. of $\frac{N}{2}$ hydrochloric acid, the mixture being kept boiling for one minute. It is then cooled, neutralised by the addition of 30 c.c. of $\frac{N}{2}$ NaHO and made up with water to 500 c.c. Such a solution contains 0·2 gram. of invert sugar per cent.

10 c.c. of Fehling's solution are titrated with this and thus we obtain the amount of invert sugar corresponding to 10 c.c. of Fehling's. Since invert sugar has rather a greater reducing power than dextrose, the figure thus obtained must be multiplied by 0·97 to obtain the amount of dextrose corresponding to 10 c.c. of Fehling's solution.

100. The Estimation of dextrose by Cole's Cyanide Method.

Principle. Fehling's solution is treated with a solution of potassium cyanide until the blue colour is just discharged on boiling. A solution of cuprous cyanide is thus formed.

$$\underset{\text{Cupric hydrate.}}{2\,Cu(OH)_2} + 4KCN = \underset{\text{Cupric cyanide.}}{2\,Cu(CN)_2} + 4KOH.$$

$$2\,Cu(CN)_2 = \underset{\text{Cuprous cyanide.}}{Cu_2(CN)_2} + \underset{\text{Cyanogen}}{(CN)_2}.$$

$$(CN)_2 + 2KOH = KCN + \underset{\text{pot. cyanate.}}{KOCN} + H_2O.$$

To the colourless solution thus obtained 10 c.c. of Fehling's solution are added, the mixture boiled and titrated with the dextrose solution until the blue colour is discharged. The end-point is not obscured by a deposit of cuprous oxide owing to the latter forming with the cuprous cyanide a double cuprous potassium cyanide which is a soluble colourless compound.

Reagents required. (i.) A solution of potassium cyanide, about 4 per cent. (Potassium cyanide is a **deadly poison** and must be handled with the greatest care. It should always be measured by means of a burette, never by a pipette.) (ii.) Fehling's solution freshly prepared according to the directions given in Ex. 63.

Apparatus required.

A 50 c.c. burette, graduated in tenths of a c.c., is connected by a short length of rubber tubing with a glass tube passing through the stopper of a 150 c.c. flask. Another hole in the stopper (rubber or cork) allows the passage of an exit tube, bent at a right-angle, for the escape of the steam. Care should be taken to have this exit-tube directed away from the experimenter. The lower end of the exit-tube is flush with the bottom of the stopper: the inlet tube, which must be drawn out to a rather fine point, should extend about half-an-inch into the flask. The tubing connecting the flask and the burette is securely fastened at both ends and is fitted with a pinch-cock or similar contrivance. The burette is held by a metal clamp, and the flask hangs down from it, there being no need to support this by a tripod, provided that the stopper fits tightly.

Methods of Analysis.

1. Fill the burette with the sugar solution: allow some to escape by pinching the stopcock and see that

all the air is displaced from the rubber connection. In the flask place 10 c.c. of Fehling's solution. Boil and whilst boiling add the cyanide solution from a burette until the blue colour is *just* discharged. Should too much cyanide be inadvertently added, a few drops of Fehling's solution can be dropped in to restore the blue tinge and the process repeated. It is necessary to keep the mixture at the boiling point the whole time. If the colourless mixture be allowed to cool, a blue tinge is generally acquired, due to the oxidation of the cuprous cyanide.

Add another 10 c.c. of Fehling's solution, fit the flask firmly to the burette and read the level of the dextrose solution in the latter. Boil, and whilst boiling add the solution slowly. The blue colour fades and the fluid becomes bluish-green. Determine the exact point when the green tinge is turned to a yellow, by the following procedure. When near the end-point, read the burette, add two or three drops of the solution and observe if the change takes place. If not, read again and repeat until all trace of the green has vanished and the mixture is definitely yellow.

Should the amount of sugar solution added be less than 5 c.c., it must be diluted so that about 10 c.c. will be required. Thus, if 1·5 c.c. are necessary, dilute $\frac{10}{1{\cdot}5} = 7$ times, *i.e.* to 10 c.c. of the dextrose solution add water to make the volume up to 70 c. c. Mix well, wash out the burette, and repeat the process with this diluted solution.

2. The amount of potassium cyanide required for 10 c.c. of Fehling's solution is 0·24 gram. If the strength of the cyanide solution be known such a volume of it as contains 0·24 gram. can be added to 20 c.c. of Fehling's solution and the mixture boiled and titrated directly with the dextrose solution.

To determine the strength of the potassium cyanide measure 10 c.c. into a large beaker, add about 100 c.c. of water, a few drops of a strong solution of sodium chloride and titrate with a standard solution of silver nitrate until a faint permanent opalescence is obtained.

The standard silver solution is prepared by dissolving 26·154 grams. of the fused salt in water and making the volume up to 1 litre. 1 c.c. = 0·02 gram. KCN.

If 10 c.c. KCN require 21·4 c.c. Ag NO_3

Then 10 c.c. KCN contain ·428 gram. KCN

So there is ·24 gram. KCN in $\frac{\cdot 24}{\cdot 0428} = 5{\cdot}65$ c.c. KCN solution.

Calculation of results and example.

10 c.c. of Fehling's solution are reduced by ·05 gram. dextrose.

1st titration required 1·1 c.c. Solution diluted 9 times.

2nd titration required 9·5 c.c.

9·5 c.c. of diluted solution = ·05 gram. dextrose.

9·5 c.c. of original " = 9 × ·05 " "

100 c.c. " " = $\frac{9 \times \cdot 05 \times 100}{9{\cdot}5}$ "

= 4·7 grams.

101. **The estimation of cane-sugar.**

Treat 40 c.c. of the solution with 30 c.c. of $\frac{N}{2}$ hydrochloric acid keeping the mixture boiling for 1 minute. Cool, neutralise by adding 30 c.c. of $\frac{N}{2}$

sodium hydrate, cool to 15°C. and make the volume up to 100 c.c. Estimate the amount of invert sugar in this solution by either of the methods given in the two previous exercises.

Calculation of results.

10 c.c. of Fehling's solution = ·45 gram cane sugar. Note the dilution of the original solution and calculate its strength on the above basis.

CHAPTER III.

THE PROPERTIES AND DIGESTION OF THE FATS.

102 (*a*) Carefully allow a drop of neutral olive oil to fall gently on to the surface of some ·25 per cent. Na_2CO_3 contained in a watch-glass. The drop of oil remains quite clear and forms a thin circular film on the surface.

(*b*) Shake 5 c.c. of neutral oil with 3 drops (only) of oleic acid in a dry test tube. With a drop of this mixture repeat (*a*) using a fresh watch-glass full of Na_2CO_3. The rancid oil slowly spreads out in an amoeboid fashion and becomes converted into a milky emulsion.

(*c*) To the remainder of the mixture of oil and oleic acid add 12 more drops of oleic acid, shake well and repeat the experiment. The drop becomes white and opaque, but does not become emulsified.

NOTES—1. It is absolutely essential that the oil be quite neutral, and this can best be tested by dropping it on to ·25 per cent. Na_2CO_3. If a spontaneous emulsion is formed, a fresh sample must be obtained, or melted fresh butter substituted.

2. The spontaneous emulsion in (*b*) is caused by the trace of oleic acid dissolving in the alkali to form a soap, diffusion currents being thus set up which divide the fat into microscopic droplets.

3. In (*c*) the large excess of oleic acid leads to the opaque ring of soap being formed round the oil, and this soap, being only slightly soluble in water, prevents the formation of an emulsion.

103. Preparation of a solution of steapsin (lipase), the fat-splitting ferment.

A perfectly fresh pig's pancreas is freed from fat, weighed, finely minced, and ground with sand. It is then extracted for 24 hours with a mixture consisting of 90 parts of pure glycerine and 10 parts of 1 per cent. Na_2CO_3, 10 c.c. of the mixture being taken for every gram. of pancreas. The fluid is strained through muslin or linen. The steapsin is destroyed as soon as the fluid becomes acid, which is usually after about three days. The extract should be kept in a refrigerator when not in use.

104. Shake 5 c.c. of neutral olive oil in a test tube with 2 c.c. of the glycerine extract of the pancreas and place the tube in a water-bath at 37°C. At the end of every ten minutes pipette off a little of the oil that rises to the surface, allow a drop of it to fall gently on to some ·25 per cent. Na_2CO_3 contained in a watch-glass, return the rest to the tube, shake vigorously and return it to the warm bath. As the action of the ferment proceeds spontaneous emulsion will occur, showing that some of the neutral oil has been converted into a fatty acid. If the action is allowed to proceed considerably further no emulsion will be produced, for the reasons stated in the notes to Ex. 94.

NOTE.—This is one of the most convenient ways of demonstrating the fat-splitting power of steapsin, but, naturally, it can only be used when perfectly neutral olive oil can be obtained.

105. Repeat the above experiment, but boil and then cool the 2 c.c. of glycerine extract before adding the olive oil. A spontaneous emulsion is not formed at any stage, showing that the ferment is destroyed.

NOTES—1. This or a similar control experiment should always be performed side by side with the actual experiment when investigating the action of ferments.

2. Be particularly careful to cool the extract after boiling, otherwise the alkali may exert a slight saponifying action at the higher temperature.

3. Instead of boiling the extract to destroy the ferment, 2 c.c. of pure glycerine might be used with the olive oil as a control.

106. Boil 10 c.c. of fresh milk, cool it under the tap, add 2 or 3 c.c. of the glycerine extract and enough litmus solution to give a marked colour. Shake well and divide into two portions, A and B. Boil A to destroy the ferment. Place both tubes in the water bath at 37°C. In the course of half-an-hour or so the blue colour in tube B will change to red, indicating that some of the neutral fat in the milk has been hydrolysed to a fatty acid.

NOTE.—The fat of milk being finely emulsified offers a very large surface for the action of the steapsin. The milk should be boiled first to destroy any bacilli present that might form lactic acid from the lactose.

107. Shake a few drops of olive oil with 5 c.c. of ether in a dry tube. The oil completely dissolves. Repeat the experiment with alcohol instead of ether. The oil dissolves partially, but is not so soluble in alcohol as in ether. Pour the alcoholic solution into water. The fat is precipitated as an emulsion.

108. Heat a few drops of glycerine with solid potassium hydrogen sulphate in a porcelain dish. A pungent odour of acrolein (acrylic aldehyde) is evolved.

$$\underset{\text{Glycerine.}}{CH_2OH \cdot CHOH \cdot CH_2OH} = \underset{\text{Acrolein.}}{CH_2 : CH \cdot CHO} + 2H_2O$$

109. Treat about 5 c.c. of a 5 per cent. solution of borax with sufficient of a 1 per cent. alcoholic solution of phenol-phthalein to produce a well-marked red colour. Add a 20 per cent. aqueous solution of glycerine, drop by drop, until the red colour is just discharged. Boil the solution: the colour returns, provided that an excess of glycerine has not been added (**Dunstan's test for glycerine**).

NOTES—1. Any ammonium salt will discharge the colour, but in this case it does not return on heating.

2. Any polyhydric alcohol is likely to give the same reaction. The sugars are all polyhydric alcohols, but are distinguished from glycerine by their reducing properties, etc., and by the fact that they are not volatile when distilled by steam.

3. The reaction at presents lacks a satisfactory explanation.

110. Shake a few drops of oleic acid with 5 c.c. of water, ether, and alcohol respectively in separate tubes. The acid is insoluble in water, but soluble in alcohol or ether.

111. Heat about 10 drops of oleic acid with 10 c.c. of water and to the hot mixture add 40 per cent. NaOH drop by drop till the solution is clear. If an excess be added the excess of sodium ions causes a precipitate (see note below). A clear solution of a soap, sodium oleate, is formed. Divide this into three portions. To A add a few drops of strong HCl or H_2SO_4 till the reaction is distinctly acid. Oleic acid separates out and rises to the surface of the tube.

To B add finely-powdered sodium chloride and shake. The soap is rendered insoluble and rises to the surface.

To C add some calcium chloride. A precipitate of an insoluble soap, calcium oleate, is produced.

NOTE.—B illustrates the principle of "salting out," which is used in the manufacture of soaps. The excess of sodium ions in the solution, produced by the addition of the sodium chloride, lowers the solubility of the sodium oleate, which is therefore precipitated.

112. Boil 5 c.c. of olive oil with 10 c.c. of a 20 per cent. alcoholic solution of sodium hydrate in a basin over a *small* flame for five minutes or until the alcohol has all evaporated away. Add about 10 c.c. of alcohol and heat again to dryness, stirring the whole time. Add about 10 c.c. of water, boil, mix thoroughly and carefully add 25 per cent. sulphuric acid till the mixture is acid. A precipitate of oleic acid is formed. Filter this off through a wet filter paper. Exactly neutralise the filtrate to litmus and apply Dunstan's test for glycerine (Ex. 109). Glycerine is present.

NOTE.—Fats are hydrolysed (saponified) by boiling soda (best in alcoholic solution) to fatty acid and glycerine. The fatty acid is converted to a soluble soap by the alkali present, but can be precipitated by acidification.

$$\underset{\text{Olive Oil.}}{C_3H_5 . (C_{17}H_{33} . COO)_3} + 3\,NaHO = \underset{\substack{\text{A soluble soap}\\ \text{(Sodium oleate).}}}{3\,C_{17}H_{33} . COONa} + \underset{\text{Glycerine.}}{C_3H_5(OH)_3}$$

CHAPTER IV.

THE CHEMISTRY OF SOME FOODS.

A. Milk.

113. Examine a drop of fresh cow's milk under the microscope with a high power. Notice the highly-refractive fat globules of varying size, the smallest globules exhibiting the peculiar vibration known as Brownian movement.

114. Take the specific gravity of milk with a lactometer. It varies between 1028 and 1034.

NOTE.—When the milk is skimmed the specific gravity rises from 1033 to 1037, owing to the removal of the fat which has a low specific gravity.

115. Take the reaction to litmus. It is faintly alkaline.

116. Take 5 c.c. of milk in a test tube and dilute with distilled water till the test tube is nearly full. Add three drops of strong acetic acid and mix thoroughly. A flocculent precipitate of caseinogen is formed, which mechanically carries the fat down with it. Filter this off and label the filtrate A. Precipitate two more portions of 5 c.c. each, adding the filtrates to A, and reserving the precipitate.

117. Take 5 c.c. of milk, add water as before, and then twelve drops of strong acetic acid. A precipitate is not produced, owing to the solubility of caseinogen in an excess of acid.

118. Treat a portion of the precipitate from Ex. 116 with some 2 per cent. Na_2CO_3 solution. The caseinogen dissolves, leaving the fat in suspension. Apply the protein colour reactions to the solution: all, except the sulphur test, are given.

119. Treat 5 c.c. of milk with 5 c.c. of saturated $(NH_4)_2SO_4$ solution. The caseinogen is precipitated, entangling the fat with it. Filter off, and boil the filtrate. A heat coagulum of lact-albumin is obtained. Treat the precipitate of caseinogen and fat on the paper with water. The caseinogen dissolves.

120. Treat a considerable portion of the precipitate obtained in Ex. 116 as directed in Ex. 44. Phosphorus is found to be present in the caseinogen.

121. Allow another portion of the precipitate obtained in Ex. 116 to drain thoroughly, press it with dry filter paper and transfer it to a dry tube. Shake it vigorously with 5 c.c. of ether, pipette off the ether, evaporate it on a basin over a water bath. A small amount of fat is left in the dish. Wipe the dish round with a piece of writing-paper. A translucent grease spot is formed.

122. Examine filtrate A. Add a drop of litmus, and note that it is markedly acid. Boil, and whilst

boiling add 2 per cent. Na_2CO_3, drop by drop, until the reaction is only faintly acid. If the reaction should, by accident, be made alkaline, dilute acetic acid must be added till the reaction is faintly acid. A coagulum of a protein known as lact-albumin is formed. Filter this off and reserve the filtrate (B).

123. Boil a small portion of filtrate B with a little Fehling's solution. A well-marked reduction is obtained, due to the presence of lactose.

124. Treat the remainder of filtrate B with two or three drops of strong ammonia and boil. A slight precipitate of calcium phosphate is produced. Filter this off, dissolve it in a little strong acetic acid, add potassium oxalate. A white precipitate of calcium oxalate is formed. Treat with 2 c.c. of nitric acid and 5 c.c. of ammonium molybdate solution. Boil for two minutes. A yellow crystalline precipitate is formed, showing the presence of phosphates in milk.

B. The Clotting of Milk.

125. Treat 5 c.c. of milk with about 2 c.c. of an active solution of rennet-ferment. Place the tube in the warm bath, and observe it from time to time. Note that the milk soon forms a clot so firm that the tube can safely be inverted: on standing longer the clot contracts and exudes a nearly clear fluid (whey).

126. Perform a control test by boiling and then cooling the rennet before adding it to the milk. Clotting does not take place.

127. Treat 5 c.c. of milk with 2 c.c. of 2 per cent. Na_2CO_3 and the same amount of rennet: place the tube in the warm bath. Clotting does not take place.

NOTES—1. Rennet can be prepared by extracting the mucous membrane of the fourth stomach of a sucking calf with glycerine. It can also be obtained by making an extract of the mucous membrane of the stomach of a pig. For class purposes a commercial preparation of rennet is most convenient.

2. In investigating the activity of a solution it is important to neutralise the solution carefully, should it be acid. Otherwise the acid itself will precipitate the caseinogen, and obscure the true clotting.

3. Rennet is destroyed by alkalies.

4. The clot (the curd) consists of the casein that has been formed from the caseinogen of the milk, together with the fat which is entangled in it.

128. Take 10 c.c. of milk, add one-third its volume of 1 per cent. potassium oxalate and divide it into three equal portions which are placed in three test tubes labelled A, B, and C.

To A add 1 c.c. of 2 per cent. calcium chloride.

To about 6 c.c. of rennet add 2 c.c. of 1 per cent. potassium oxalate: divide into three portions. Add a portion to A, another to B: boil, and then cool the third portion and add it to C.

Place the three tubes in the warm bath for about ten minutes. Note that A clots and that B and C do not.

To B and C add 2 c.c. of 2 per cent. $CaCl_2$. A flocculent precipitate of insoluble casein is produced in B, but not in C.

NOTES—1. The clotting of milk by rennet takes place in two stages.

(*a*) The caseinogen is converted to soluble casein by the ferment.

(*b*) The soluble casein is acted on by a soluble calcium salt to form insoluble casein.

2. In the exercise given A contains rennet and $CaCl_2$, and therefore clots. B contains rennet but no soluble calcium salt; soluble casein is formed, which is precipitated by the subsequent addition of an excess of $CaCl_2$. C contains no rennet, and therefore suffers no change, caseinogen not being precipitated by $CaCl_2$.

C. Cheese.

129. Shake some grated cheese in a dry test tube with ether, and examine the ethereal solution for fat as in Exercise 121. Fat is present in considerable quantity.

130. Pound the residue from the above in a mortar with a 2 per cent. solution of sodium carbonate and filter. Acidify a portion of the filtrate. A precipitate of casein is formed, which is soluble in excess of acid. To the remainder of the filtrate apply the usual protein colour reactions: they are all obtained.

D. Potatoes.

131. Scrape the clean surface of half a potato with a pen-knife, keeping the scrapings as fine as possible. Place the scrapings in a beaker of water, stir well, and strain through fine muslin into another beaker. Allow this to stand for a few minutes and then note the white deposit of starch. Pour off the supernatant fluid and reserve it for the next exercise. Fill the beaker containing the starch with water, stir well, and again allow the starch to settle. By repeating this process of lixiviation the starch can be obtained quite pure.

Examine a little microscopically and note the characteristic form of the grains (See Ex. 75). Heat a little with water, cool, and add iodine. A deep blue colour is obtained.

132. Filter the fluid A, and test portions of the filtrate for proteins by the usual colour tests. Only small quantities of protein are found to be present.

E. Flour.

133. Mix some wheat flour with a little water to form a stiff dough. Allow this to stand for a short while (heat for about ten minutes on a warm bath at 37°C.). Wrap a piece, the size of a chestnut, in muslin, and knead it for a few minutes in a basin of water; pour this into a beaker, and note the white deposit of starch grains that settles down on standing. Examine this microscopically, noting that the grains differ considerably from those of potato-starch in being smaller, circular, with a central hilum. Make a drawing of the grains. Boil a little with water, cool, and add a drop of iodine. The deep blue starch reaction is obtained.

134. Knead the dough thoroughly under the tap until no more starch comes through the muslin. A yellowish, sticky mass, known as gluten, is left behind. Test portions of this by the usual protein colour reactions: they are all obtained, gluten being a protein.

NOTE.—Gluten does not exist preformed in flour, but is formed from its precursors (globulins) on the addition of water. It is best to let the dough stand for a short while in a warm place to allow this process to be completed.

F. Bread.

135. Take a piece of the crumb of a stale white loaf, rub it up finely and pound with cold water in a mortar. Strain and squeeze through muslin. A white fluid is obtained containing wheat starch grains. Filter the fluid. To a portion of the filtrate add a little Fehling's solution and boil: a well-marked reduction occurs due to the presence of dextrose. To another portion add iodine: a purple colour is produced, showing the presence of erythro-dextrin. If very dilute iodine be cautiously added, a blue colour is produced at first, showing that a small amount of soluble starch is present.

Boil a small amount of the residue of the bread with water in a beaker, strain through muslin and filter. Cool and test the filtrate for starch and dextrin (Ex. 86, 87 and 98).

136. Repeat the above exercise, using the crust of bread instead of the crumb. Note that dextrose is absent or present in traces only: dextrin and starch are present, a considerable portion of the latter existing as soluble starch and being present in the cold water extract.

NOTE.—The sugar and dextrin present arise from the action of the ferments of the yeast that is employed in making the bread. The absence of dextrose from the crust is due to its conversion into caramel in the baking.

CHAPTER V.

THE DIGESTION OF STARCH AND PROTEIN.

A. The Properties and Action of Saliva.

137. Collect about 5 c.c. of your own saliva in a small beaker. Test the reaction with neutral litmus paper: it is alkaline.

NOTE.—The first portion of saliva collected is very apt to be neutral or even slightly acid, probably owing to bacterial decomposition in the mouth. But if the secretion is free, that collected later is invariably alkaline.

138. Transfer the saliva to a test tube and add strong acetic acid. A stringy precipitate of mucin is formed, insoluble in excess of acid. Stir the mixture vigorously with a glass rod: the mucin forms a clump which can be removed by the rod. To the clear fluid remaining add some Millon's reagent and boil. Only a slight red precipitate is formed, showing that the proteins of saliva consist almost entirely of mucin.

139. Obtain diluted saliva as follows: warm some distilled water in a beaker to about 40°C. With a portion of this thoroughly rinse the mouth out. Now take about 20 c.c. of the warm water into the mouth

and move it about by the tongue for at least a minute. Collect the fluid thus obtained in a clean beaker, and repeat the process twice more. Thoroughly mix the diluted saliva thus obtained, shake it vigorously and filter.

140. In a clean test tube take 5 c.c. of 1 per cent. starch paste, freshly prepared with distilled water, and 5 c.c. of the diluted saliva. Mix well and place the tube in a water-bath maintained at a temperature of about 40°C. Place a series of drops of iodine solution on a white porcelain plate, and from time to time transfer, by means of a glass rod, a drop of the digesting mixture to a drop of the iodine. The blue colour produced at first will later become a blue-violet, red-violet, red-brown, and a light-brown yellow colour, as the starch, and then the erythro-dextrin are converted to other products. When a drop of the mixture no longer gives any colour with iodine, boil a few c.c. of it with a few c.c. of Fehling's solution. A well-marked reduction is obtained, showing that a ferment (ptyalin) in saliva has converted the starch into a reducing sugar, which is, however, not dextrose, but maltose.

141. Perform a control test by first boiling, and then cooling the saliva before adding it to the starch (See Ex. 105). No action whatever takes place when the mixture is allowed to stand on the warm bath, proving that the effect in the above exercise was due to a ferment.

142. **The investigation of the activity of ptyalin under various conditions by the method of the achromic point.**

In each of a series of clean test tubes place about 1 c.c. of an iodine solution that has been diluted to a pale straw-colour with distilled water.

Carefully measure 5 c.c. of the 1 per cent. starch paste into a perfectly clean test tube, add five drops of distilled water and place the tube in a warm bath at 40° C. for a few minutes.

To the starch paste add 5 c.c. of the diluted saliva, previously warmed to 40° C. in the warm bath. Mix the two fluids, and note the time of the addition. At intervals transfer a few drops of the digestive mixture to one of the samples of iodine by means of a small pipette made from quill tubing. The same series of colour changes will be observed as were seen in Ex. 140. Note the time when the addition ceases to produce any colour. This point, which is the moment when the last trace of erythro-dextrin is converted to achroo-dextrin and maltose, is known as the achromic point. The time that is taken to reach this point ("chromic period") is a measure of the activity of the ferment.

Repeat the exercise, and note that the chromic period obtained agrees fairly closely with that previously found.

NOTES—1. It will be seen that the starch and the ferment solution are separately warmed to the temperature at which the exercise is

performed. Otherwise the results will not be strictly comparable with those of the following exercises.

2. A convenient chromic period is one of about five minutes. If it is less than two minutes, the saliva should be diluted with an equal volume of distilled water. If it is more than ten minutes the starch paste should be diluted with an equal bulk of water and boiled well to ensure thorough mixing. In either case the chromic period under these new conditions must be carefully noted for comparison with those obtained in the following exercises.

143. Repeat the above exercise, substituting five drops of a 5 per cent. solution of sodium chloride for five drops of water. The chromic period is considerably reduced.

NOTE.—The concentration of NaCl in the digestive mixture is between ·01 and ·02 per cent. Even lower concentrations than this have a marked effect in increasing the activity of ptyalin. A concentration of 5 per cent. of NaCl usually slightly decreases the activity.

144. Repeat the above exercise using one drop of ·4 per cent. hydrochloric acid and four drops of water. The chromic period is considerably reduced. With two drops of ·4 per cent. HCl and three drops of water the chromic period may or may not be reduced, according to the alkalinity of the saliva.

NOTE.—One drop of ·4 per cent. HCl in the mixture gives a concentration of acid of about ·002 per cent. But the saliva contains a little alkali and also some protein which enters into a loose combination with the acid. The concentration of free HCl in the digestion mixture will therefore be less than ·002 per cent.

145. Repeat the above exercise, using five drops of ·4 per cent. hydrochloric acid instead of one drop. The chromic period is indefinitely prolonged.

NOTE.—The concentration of HCl in this experiment is under ·02 per cent. In the absence of proteins such a concentration rapidly destroys ptyalin, the activity not returning on neutralisation.

146. Repeat Exercise 140 at the temperature of the room, at 30° C. and at 55° C. The chromic period is least at 40° C.

B. The Action of Pepsin on Proteins.

For the following experiments use a 1 per cent. solution of commercial pepsin in water. Note that the solution is acid to litmus.

147. Place equal amounts of fresh washed fibrin in four test tubes labelled A, B, C, and D.

To A add 5 c.c. of pepsin and 5 c.c. of ·4 per cent. HCl.

To B add 5 c.c. of pepsin and 5 c.c. of water.

To C add 5 c.c. of water and 5 c.c. of ·4 per cent. HCl.

To D add 5 c.c. of pepsin that has been boiled and then cooled, and 5 c.c. of ·4 per cent. HCl.

Place the four tubes in a water bath at 40° C. for at least thirty minutes.

Note that in

A, the fibrin swells up, becomes transparent and then dissolves;

B, the fibrin is unaltered;

C, the fibrin swells up, becomes transparent, but does not dissolve;

D, the fibrin is like that in C.

NOTE.—These exercises show that neither ·2 per cent. HCl alone, nor pepsin alone, can digest fibrin, but that pepsin in the presence of ·2 per cent. HCl has this property. In D the ferment pepsin has been destroyed by boiling.

148. Collect the fibrin from test tubes B, C, and D and add it to A. Replace A on the water-bath for another thirty minutes and then filter. Add a drop or two of litmus to the filtrate, and then carefully neutralise it with dilute alkali. A precipitate of meta-protein is formed. Filter this off and examine the filtrate for albumoses (Exs. 50 and 51).

NOTES—1. If the digestion is allowed to proceed for a considerably longer period (varying with the activity of the pepsin) the meta-protein and albumoses are converted to peptones.

2. All samples of commercial pepsin contain peptones, so that the detection of peptone after a digestion experiment is no proof of the activity of the pepsin.

149. Obtain some fibrin that has been stained with carmine (see note, below) and repeat Ex. 147 with this instead of with the fresh fibrin. After half-an-hour's digestion note that only in A has the protein dissolved, this being readily seen by the fluid becoming tinged with red.

NOTE.—The carmine solution for staining fibrin is prepared by dissolving 1 gram. of carmine in about 1 c.c. of ammonia and adding 400 c.c. of water. The solution is kept in a loosely-stoppered bottle till the smell of ammonia has become faint. Fresh washed fibrin is chopped finely, placed in the carmine solution for twenty-four hours, strained off and washed in running water till the washings are colourless. If not required immediately, it should be kept under ether and washed with water before use. It cannot be used for testing for trypsin, owing to the solubility of the dye in alkalies.

150. Treat 5 c.c. of the pepsin solution with half its volume of 2 per cent. sodium carbonate and place on the bath at 40° C. for half-an-hour. Neutralise with ·4 per cent. HCl, and then add an equal volume of ·4 per cent. HCl to the fluid. Add some fibrin and

place the tube on the warm bath. The fibrin does not dissolve, showing that pepsin is destroyed by dilute alkaline salts.

C. The Action of Trypsin on Proteins.

For the following exercises a commercial extract of the pancreas, known as Benger's "Liquor pancreaticus," can be employed. It should be diluted with an equal bulk of distilled water.

151. Place equal amounts of fresh washed fibrin in four test tubes labelled A, B, C, and D.

To A add 5 c.c. of trypsin and 5 c.c. of 2 per cent. Na_2CO_3.

To B add 5 c.c. of trypsin and 5 c.c. of water.

To C add 5 c.c. of 2 per cent. Na_2CO_3 and 5 c.c. of water.

To D add 5 c.c. of trypsin that has been boiled and then cooled, and 5 c.c. of 2 per cent. Na_2CO_3.

Place the four tubes in a warm bath at 40° C. for half-an-hour to an hour, and note that in A the fibrin dissolves, but is unaltered in B, C, and D.

152. **The Products of the Action of Trypsin on Proteins.**

150 grams. of commercial casein ("protene" or "plasmon"), 125 to 150 c.c. of Benger's "Liquor pancreaticus," and a litre of 1 per cent. Na_2CO_3 have been digested for about ten days at 40° C. in a large flask, 1 gram. of sodium fluoride and about 30 c.c.

of chloroform or toluol being added, and the mouth of the flask securely plugged with cotton wool, soaked in chloroform, to prevent bacterial decomposition. About 100 c.c. of the mixture are given to you. Boil the mixture, and whilst boiling add strong acetic acid, drop by drop, till the reaction is acid. Cool under the tap, and filter off the undigested casein, etc.

A. Treat 5 c.c. of the filtrate with bromine water, drop by drop; a pink colour gradually develops, which deepens and then disappears as more bromine water is added. When the colour is no longer intensified by the addition of bromine, add 2 or 3 c.c. of amyl alcohol and shake. On standing, the alcohol rises to the surface coloured a fine red or violet. This reaction is due to the presence of *tryptophane*.

B. Treat another 5 c.c. of the filtrate with ten drops of concentrated sulphuric acid and 10 c.c. of a 10 per cent. solution of mercuric sulphate in 5 per cent. H_2SO_4. Shake the tube and leave it for five minutes. Note the yellow precipitate of a mercury compound of *tryptophane*. Filter this off and label the filtrate A. Wash the precipitate through a hole in the paper into a clean tube, fill with water, shake and filter again, neglecting the filtrate. Wash the precipitate on the paper once more with water and then let it drain. Scrape a portion off the paper, transfer it to a tube, add 2 c.c. of "reduced oxalic acid" and then 2 c.c. of concentrated sulphuric acid. A purple colour is produced, showing that tryptophane is responsible for the glyoxylic reaction (See Ex. 3).

Treat another portion of the precipitate with Millon's reagent and boil. A yellow colour is produced, not the characteristic red of Millon's reaction.

To another portion of the precipitate apply the xanthoproteic test. A well-marked reaction is obtained (See notes to Ex. 1).

To portions of filtrate A apply the glyoxylic, Millon's, and the xanthoproteic reactions. Only the latter two are obtained, the tryptophane, but not the tyrosin, having been removed by the mercury reagent employed.

C. Treat the remaining 90 c.c. of the filtrate with a few drops of ammonia and evaporate to a small bulk (about 20 c.c.) either on the water-bath or by use of a small free flame. Allow the residue to stand twenty-four hours. Notice the formation of a crystalline crust. Examine a portion of this microscopically and observe the feathery masses and sheaves of fine white needles, characteristic of *tyrosin*. Filter this off and evaporate the filtrate still further. *Leucin* separates out on standing, and, examined microscopically, shows rounded cones with a radiating striation. Make a drawing of the crystals of tyrosin and leucin.

D. The Action of Amylopsin on Starch.

153. Repeat Exercise 140 using diluted "liquor pancreaticus" in place of the diluted saliva. The starch is rapidly digested and converted to maltose by the action of the ferment amylopsin.

E. Tests for Hydrochloric and Lactic Acids.

154. **Gunsberg's reaction for hydrochloric acid.** Dilute some ·4 per cent. HCl with nine times its volume of water, thus obtaining ·04 per cent. HCl. Place a few drops of this in a small porcelain dish, add a few drops of Gunsberg's reagent and carefully evaporate over a very small flame. The residue is of a beautiful bright red when even ·005 per cent. of HCl is present. Otherwise the colour is yellow or brownish.

NOTES—1. Gunsberg's reagent consists of two parts of phloroglucin, one part of vanillin, and thirty parts of 95 per cent. alcohol.

2. This is the best test for free hydrochloric acid in gastric juice, the other tests being given by organic acids when concentrated.

155. **Uffelmann's reaction for lactic acid.** Treat a few c.c. of Uffelmann's reagent with a few c.c. of a dilute (0·4 per cent.) solution of lactic acid. The violet colour is instantly turned to a yellow.

NOTES—1. Uffelmann's reagent is prepared by treating a 1 per cent. solution of phenol (carbolic acid) with very dilute ferric chloride till the solution becomes of amethyst-violet colour.

2. The reaction is not very reliable, since other acids decolourise the solution. Even very dilute lactic acid, however, gives the characteristic yellow colour, which is to be carefully distinguished from a mere decolourisation.

156. **Hopkins' reaction for lactic acid.** To 3 drops of a 1 per cent. alcoholic solution of lactic acid in a clean, *dry* test tube add 5 c.c. of concentrated sulphuric acid and 3 drops of a saturated solution of copper sulphate. Mix and place the tube in a beaker of boiling water for about five minutes. Cool

thoroughly under the tap, add two drops of a ·2 per cent. alcoholic solution of thiophene, and shake. Replace the tube in the boiling water. As the mixture gets warm, a fine cherry-red colour develops.

This test can be applied as follows to detect the formation of lactic acid when muscle is stimulated. Pith a frog and remove one hind limb. Expose the lumber plexus of the other side and stimulate it for ten minutes by means of a strong interrupted current. Cut off the limb, strip the skin off the two limbs, and treat the thigh muscles separately in the following manner. Pound in a mortar with sand, and then with 15 c.c. of 95 per cent. alcohol. Transfer the mixture to a beaker and warm in the water bath for a few minutes. Filter through a small paper and evaporate the filtrate to complete dryness on the water bath. Treat the residue with about 5 c.c. of cold water and rub it up thoroughly with a glass rod. Filter and boil the filtrate in a test tube for about a minute with as much animal charcoal as will lie on a threepenny-piece. Filter again, and evaporate the filtrate to complete dryness on the water bath. Allow the residue to cool, treat it with 5 c.c. of concentrated H_2SO_4, and shake round till solution is obtained. Transfer to a *dry* test tube, add 3 drops of saturated $CuSO_4$, and heat in boiling water for five minutes. Cool thoroughly, add 2 drops of thiophene solution, and replace the tube in the boiling water. A fine red colour develops in the tube containing the extract from tetanised muscle, but not in the other.

CHAPTER VI.

THE COAGULATION OF BLOOD.

157. **Preparation of fibrin ferment (thrombin).** Blood serum is treated with four or five times its volume of strong alcohol, well stirred and allowed to stand for two or three days. The precipitate is collected, dried on filter paper in the air, and extracted with water. The filtered extract contains fibrin ferment.

158. **Preparation of "salted" plasma.** Two litres of water are placed in a large bottle or jar (provided with a tightly-fitting stopper) and the level of the fluid marked by a label. The water is poured off and 400 c.c. of a saturated solution of magnesium sulphate substituted. Blood is collected in the bottle till the level is reached, care being taken to ensure thorough mixing with the salt solution by stopping the flow of blood from time to time and turning the bottle upside down. The corpuscles are removed by centrifugalisation and the plasma pipetted off. It should be kept in a refrigerator till required.

159. **The clotting of salted plasma.** Take 2 c.c. of salted plasma in a test tube, add 10 c.c. of water, and divide into two portions, A and B. To A add a few drops of fibrin ferment (or of serum). Place both tubes in the warm bath at 40°C. and examine from time to time. Clotting takes place in both tubes, but much more rapidly in A than in B.

160. **The preparation of fibrinogen.** To 20 c.c. of the salted plasma add an equal volume of a saturated solution of sodium chloride. A precipitate of fibrinogen is formed. Allow the tube to stand for a few minutes

and then filter through a small paper. Scrape the precipitate off the paper and treat it with about 5 c.c. of 5 per cent. NaCl. The fibrinogen dissolves. Divide the solution into two portions, C and D. To C add two drops of fibrin ferment. Place both tubes in the warm bath and observe them at intervals. C clots rapidly; D very slowly.

161. **The heat-coagulation of fibrinogen.** Heat 5 c.c. of salted plasma as in Ex. 9. Notice the coagulation of fibrinogen which occurs at 56°C. Continue heating to 60°C. and then filter. Dilute the filtrate as in Ex. 159; add fibrin ferment, and place on the warm bath. Coagulation does not occur.

NOTE.—Fibrinogen is a globulin, but differs from other proteins of this class in being precipitated by half-saturation with NaCl. The precipitate obtained by saturating plasma with NaCl consists of a mixture of fibrinogen and serum-globulin which is known as plasmine.

162. **Preparation of oxalate plasma.** Blood is drawn into a bottle as in Ex. 158, which has 200 c.c. of a 1 per cent. solution of potassium oxalate in place of the 400 c.c. of saturated magnesium sulphate. The plasma is separated, as before, by centrifugalisation.

163. **The clotting of oxalate plasma.** Dilute 5 c.c. of the plasma with 10 c.c. of distilled water and divide into three portions E, F, and G. To E add a few drops of 1 per cent. calcium chloride; to F, a few drops of fibrin ferment. Place the three tubes on the water bath and observe them at intervals. E clots in a few minutes; F clots slowly; G does not clot.

164. **Preparation of fluoride plasma.** This is prepared as oxalate plasma (Ex. 162), using a 3 per cent. solution of sodium fluoride in place of the 1 per cent. potassium oxalate.

165. **The clotting of fluoride plasma.** Dilute 5 c.c. with 10 c.c. of water and divide into three portions, H, K, and L. To H add a few drops of 1 per cent. calcium chloride; to K a few drops of fibrin ferment. Place the three tubes in the warm-bath and observe them at intervals. K clots rapidly; H and L do not clot.

CHAPTER VII.

OXYHAEMOGLOBIN AND ITS DERIVATIVES.

166. In each of a series of test tubes labelled A to F place a few c.c. of defibrinated blood.

Warm A to 50°C., by placing the tube in a beaker of water at that temperature.

To B add an equal volume of water and warm to 50°C.

To C add an equal volume of ·9 per cent. NaCl and warm to 50°C.

To D add a few drops of bile or of a solution of bile salts, shake, and warm to 50°C.

To E add about ten drops of ether or chloroform, shake, and warm to 50°C.

Place F in a freezing mixture of ice and salt for five minutes and then warm to 50°C.

The blood in B, D, E, and F becomes laked, *i.e.* comparatively transparent, owing to the haemoglobin of the corpuscles having been dissolved in the fluid.

NOTE.—If an excess of ether is added, the proteins are precipitated.

167. **Rapid method for preparing crystals of oxyhaemoglobin** (Reichert). To a few c.c. of defibrinated dog's blood in a test tube add ether, drop by drop, till the blood is completely laked. Add to the blood a

pinch of finely powered ammonium oxalate; allow the salt to dissolve by gentle shaking, and let the tube stand. Crystals of oxyhaemoglobin separate out, especially if the solution is cooled to 0°C. by means of ice. Examine them microscopically, and note that they are in the form of thin rhombic prisms.

Make a drawing of the crystals.

NOTE—This experiment does not always succeed as described. If the blood fails to crystallise out in an hour, place a drop on a slide, spread it out to form a thin layer and leave it for five minutes; cover with a slip and note the crystals of oxyhaemoglobin that form at the edges.

168. The use of the direct-vision spectroscope.

The instrument described is the small pocket spectroscope, with wave-length scale attached, manufactured by Zeiss and Co. The instrument (fig 1) consists of two tubes. The shorter tube A contains a transparent photographic scale of wave-lengths, with a mirror to project its image into the field of vision. By means of the tube D this scale can be focussed; and by the screw F it can be adjusted to its proper position. The tube G contains a series of alternating prisms of crown and flint glass, arranged to allow the spectrum to be observed by the eye in the line of the tube. The tube B that slides on G has a vertical slit, the width of which can be adjusted by turning the collar E.

To adjust the spectroscope: see that D and B are pushed in as far as they will go. Look through C towards the light with A to your left, and turn E till the spectrum is only just visible. (It is most important to use an extremely narrow slit). Slide B out very slowly till fine black vertical lines can be seen in the spectrum, and notice particularly a fine black line immediately to the left of the narrow strip of yellow. This line is known as the D line of Fraunhofer. The wave-length of it is $\cdot 59\mu$, a position indicated on the scale by the division marking it (the one to the right of 0·6) being produced further down than any other. If necessary alter the position of the scale by turning the screw F until the D line exactly coincides with the division mentioned. If the instrument has to be adjusted at night-time, when the D line cannot be observed, set the scale by use of the emission-spectrum of sodium (obtained by placing a few

crystals of common salt on the wick of a spirit lamp). The emission spectrum of sodium exactly corresponds to the D line. The scale is

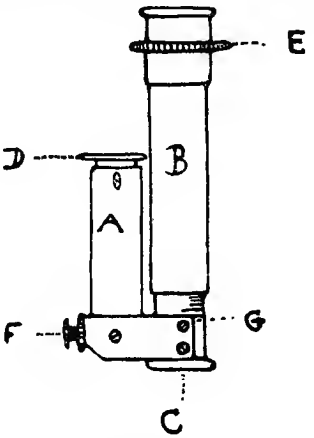


Fig. 1.—*Zeiss' direct-vision spectroscope with wave-length scale* ($\times \frac{1}{2}$). For description see text.

so drawn that, if it be set in position as described, the wave-length of light in any part of the visible spectrum can be read directly.

The numbers on the scale indicate wave-lengths in thousandths of a millimetre, the unit being 1μ. It is, perhaps, more usual to give wave-lengths in millionths of a millimetre, the unit being 1 λ. Thus the wave-length of the D line is 589 λ. The other Fraunhofer lines that can be readily observed with the instrument are C (657 λ), E (527 λ), b (518 λ) and F (486 λ).

To observe absorption spectra: slightly open the slit of the spectroscope, thus obtaining a better illumination. Direct the instrument to the light, and place the test tube, containing the fluid to be examined, directly in front of, and touching, the tube B, with its axis parallel to the slit, taking care not to interfere with the illumination of the scale. With strong solutions of certain pigments observed in this way it is often difficult to avoid illuminating the two ends of the spectrum, the light being reflected from the sides of the tubes, and not passing through the solution. To obviate this it is perhaps better to place the solution in a beaker, remembering that the absorption of light increases with the depth

of layer examined, as well as with the concentration of the pigment. For accurate work the haematoscope should be employed. This is a vessel with parallel glass sides 1 cm. apart.

In handling the instrument the screw F is very liable to be turned and so the position of the scale to be shifted. From time to time, therefore, the slit should be narrowed, and an observation made to ascertain whether any shifting of the scale in reference to the D line has occurred.

Record the absorption of light of the various pigment solutions on the blank scale, to be found towards the end of the book. Fill in with black pencil marks the exact parts of the spectrum where light is absorbed, leaving the remainder blank. It will not be found advisable to use coloured pencils.

169. **Oxyhaemoglobin.** Take 5 c.c. of distilled water in a test tube, and add one drop of defibrinated blood, shake well and observe the spectrum of **dilute** oxyhaemoglobin. There are two absorption bands in the green. The one near the D line (the α band) is somewhat narrower and darker than the β band. The middle of α is about λ 578, and that of β about λ 540.

170. Add two more drops of defibrinated blood and examine again. The spectrum has become very much cut off, especially at the blue end: the absorption bands have probably merged into one, leaving a little patch of blue light and a broader belt of red light on the two sides. Record the spectrum of the solution on the chart as that of a **medium** solution of oxyhaemoglobin.

171. Add another drop or two of defibrinated blood, and note that the blue light becomes absorbed, light only coming through in the red (**Strong** solution.) If the concentration is still further increased, the red is also absorbed.

172. **Haemoglobin (reduced haemoglobin).** Treat 5 c.c. of water with two drops of defibrinated blood and thus obtain a solution of oxyhaemoglobin of such a strength that two well-marked absorption bands can be observed. Add two drops of a solution of ammonium sulphide, mix and warm to about 50°C., avoiding any unnecessary shaking: or if Stokes' fluid is obtainable, add two or three drops, in which case there is no necessity to warm. Note, in the latter case, that the bright scarlet colour of oxyhaemoglobin gives place to the colour of reduced haemoglobin. Examine the solution spectroscopically. There is a single broad band in the green which overlaps the space enclosed by the two bands of oxyhaemoglobin, and is fainter than either. Its centre is about λ 565.

NOTE.—Stokes' fluid is prepared as follows: dissolve 3 grams. of ferrous sulphate in cold water: add a cold aqueous solution of 2 grams. of tartaric acid and make the solution up to 100 c.c. with water. Immediately before use add strong ammonia until the precipitate first produced is redissolved. It rapidly absorbs atmospheric oxygen and must, therefore, be freshly prepared. Its great advantage over ammonium sulphide is that it can be used in the cold, whilst with the sulphide the solution must generally be warmed.

173. Place your thumb over the top of the test tube containing the reduced haemoglobin and shake vigorously. Examine immediately with the spectroscope, and note that the two bands of oxyhaemoglobin have reappeared owing to the oxidation of the haemoglobin by the oxygen of the air. If the tube be allowed to stand for a short while, reduction may appear again from excess of reducing reagent present.

174. **Carboxyhaemoglobin.** Obtain some CO-haemoglobin that has been prepared by passing a stream of carbon monoxide or coal-gas through a solution of oxyhaemoglobin. Notice the peculiar bluish tinge of the solution. Examine a portion spectroscopically, and, if necessary, add water till two well-marked bands are visible. Note that they are very similar to the two bands of oxyhaemoglobin. Accurate observation, however, will show that they are both slightly nearer the violet end of the spectrum, the middle of (*a*) being λ 572 and of (*β*) λ 535.

175. Take a portion of the diluted solution of CO-haemoglobin just examined, treat it with a few drops of ammonium sulphide, warm gently and examine with the spectroscope. No change takes place in the spectrum. (Distinction from oxyhaemoglobin.)

176. In two test tubes place 2 or 3 c.c. of solutions of oxyhaemoglobin and CO-haemoglobin of the same depth of colour. Fill the test tubes with water and mix well. Note that the CO-haemoglobin takes on a well-marked carmine tint, whilst the oxyhaemoglobin turns yellow. This simple test, which can only be seen on extreme dilution, rapidly serves to distinguish the two compounds.

177. **Katyama's test for CO-Haemoglobin in blood.** Add 5 drops of blood to 10 c.c. of water; then add 5 drops of orange-coloured ammonium sulphide. Mix and add 3 drops of strong acetic acid, or as much

as may be necessary to make the mixture faintly acid. With blood containing CO, a rose-red colour appears: with normal blood a dirty greenish-grey. The colour is still perceptible with one part of the CO-blood to 5 of normal blood.

178. **Rubner's test.** To undiluted blood add 4 or 5 volumes of basic lead acetate solution and shake thoroughly for a minute. Blood containg CO becomes red: normal blood brown. On standing, the normal blood gradually becomes a chocolate colour and then a brownish grey, while CO-blood remains red.

179. **Methaemoglobin.**—To 5 c.c. of water add four drops of defibrinated blood. To the strong solution of oxyhaemoglobin thus formed add two drops of a saturated solution of potassium ferricyanide. The colour of the solution changes to a chocolate-brown. Examine with the spectroscope: there is visible a prominent band in the red, with its centre at about λ 630. There is marked absorption of the blue end of the spectrum. Dilute with an equal bulk of water and examine again: two faint bands appear in the green in the position of the bands of oxyhaemoglobin.

180. Dilute the solution of methaemoglobin thus obtained with another volume of water. Treat 5 c.c. of this with two or three drops of ammonium sulphide and examine immediately. The colour changes to a red; the absorption band in the red disappears, and the spectrum of oxyhaemoglobin is seen. Gently

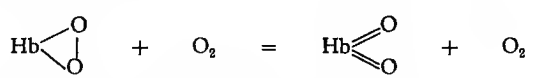
warm the solution and allow it to stand for a short time (possibly with the addition of another drop or two of the reducing reagent). The two bands give place to the single band of reduced haemoglobin. Shake with air: oxyhaemoglobin is reformed.

181. Take a few c.c. of defibrinated blood in a test tube, add an equal quantity of water and warm to 50°C. to lake the blood. To the solution thus obtained add an equal bulk of saturated potassium ferricyanide. Mix by giving one shake, and then hold the tube at rest in an oblique position for a short time. Note the bubbles of gas (oxygen) that are evolved.

NOTES—1. Oxyhaemoglobin is converted to methaemoglobin by the action of oxidising reagents, such as ferricyanides, nitrites, chlorates, and permanganates, and in the body, by the action of many aromatic substances, such as phenol.

2. The reaction is peculiar in that an amount of oxygen is evolved equivalent to that held in combination by the oxyhaemoglobin, although methaemoglobin contains the same percentage of oxygen as oxyhaemoglobin.

The reaction might be represented by the following equation.



Oxyhaemoglobin. From ferricyanide. Methaemoglobin. Gas.

The oxygen is represented as being in a different state of combination in methaemoglobin, since it cannot be removed by submitting the compound to a vacuum.

3. When methaemoglobin is treated with a reducing reagent, the first change that occurs is that the linkage of the oxygen atoms reverts to that of oxyhaemoglobin; later the oxygen is removed and reduced haemoglobin formed.

182. **Acid haematin.** To 5 c.c. of water add four drops of defibrinated blood and five drops of strong acetic acid and heat. The colour changes to brown; and the solution shows an absorption band in the red, which is further from the D line than that of methaemoglobin. Its centre is about λ 650.

183. **Acid haematin in ethereal solution.** Treat a few c.c. of defibrinated blood with one drop of strong hydrochloric acid and a few c.c. of acetic acid: extract this with about 5 c.c. of ether by gentle shaking, pour the ether into a clean tube and examine it with the spectroscope. There is a prominent band in the red (centre λ 638); on dilution with ether three other bands can be seen; a very narrow one with centre λ 582; a broad one stretching from about λ 555 to λ 530 and another from λ 512 to λ 498.

184. **Alkaline haematin.** Treat a moderately strong solution of oxyhaemoglobin with a few drops of strong sodium hydrate and warm. The colour changes to brown. Examine with the spectroscope: a faint band is seen in the red, stretching from the D line to about λ 630. There is a considerable absorption of the blue end of the spectrum.

185. **Alkaline haematin in alcohol.** Mix defibrinated blood into a thin paste with solid potassium carbonate and evaporate to complete dryness on a water bath. Powder the residue, boil with alcohol in a flask on the water-bath and filter. The solution contains alkaline haematin free from proteins. It

shows the absorption band of alkaline haematin more distinctly than the crude aqueous solution prepared in Ex. 184.

186. **Haemochromogen** (reduced alkaline haematin). Prepare a solution of alkaline haematin from dilute oxyhaemoglobin as in Ex. 184. Treat it with a few drops of ammonium sulphide. The colour of the solution changes to red. Examine with the spectroscope. Two absorption bands are seen in the green. The band nearer the D line (the α band) is very prominent and sharply defined, with its centre at about λ 558. The β band is much fainter and has its centre at λ 520.

NOTE.—In very dilute solutions only the α band can be seen. The absorption of light in this region is so intense that if a solution of oxyhaemoglobin, so dilute that its absorption bands cannot be readily seen, be converted by appropriate means into haemochromogen, the α band of this pigment is usually observable.

187. **Acid haematoporphyrin.** To a few c.c. of concentrated sulphuric acid in a test tube add two drops of defibrinated blood and mix by gentle shaking. Note the rich purple colour of the solution. Examine with the spectroscope. Two bands are seen: α band, with centre at λ 600, being fainter and narrower than β band, centre λ 554.

188. **Alkaline haematoporphyrin.** To the solution of acid haematoporphyrin just prepared add five or six more drops of defibrinated blood, shaking gently after the addition of each drop. Pour the strong solution into about 50 c.c. of cold water in a beaker, stir well and note the precipitate that rises to the

surface. Transfer this precipitate to a test tube by means of a rod; treat it with a few c.c. of alcohol and boil. Add 5 c.c. of sodium hydrate. A solution of alkaline haematoporphyrin is thus obtained, which examined spectroscopically after suitable dilutions shows a four banded spectrum. The centres of the bands are at λ 622, λ 576, λ 539, and λ 504 approximately.

NOTE.—The conversion of blood pigment to haematoporphyrin involves two processes. Firstly, the acid splits off the protein constituent (globin) and forms acid haematin; secondly, the acid haematin loses its iron and becomes haematoporphyrin. The first change is effected very readily by even dilute acids, but the separation of the iron from the haematin normally requires highly concentrated mineral acids. It has, however, been shown that if the blood be first reduced the iron is split off with much greater ease by the acid. An efficient method of reducing defibrinated blood is that of "auto-reduction," in which a tightly corked vessel full of blood is allowed to stand for a few days. If exercises 187 and 188 be carried out with this reduced blood, care being taken by use of a pipette to prevent re-oxidation, the haemoglobin is entirely converted to haematoporphyrin, no trace of the brown haematin being left.

189. **Preparation of haemin crystals.** (Teichmann's crystals). Place a drop of defibrinated blood on a glass slide, add a minute crystal of sodium chloride and rub with a glass rod till the salt has dissolved. Evaporate to complete dryness by supporting the slide about a foot above a *small* flame. Rub the red residue to a fine powder with a penknife, collect it into a little heap and add a drop of glacial acetic acid on the end of a glass rod. Rub into a paste, and place a little of this on a clean slide, add a drop of glacial acetic acid, cover with a

slip and cautiously heat over a small flame till it just boils. Let a drop more of the acid run under the slip and then allow to cool. Examine microscopically for the brown rhombic prisms of haemin (haematin hydrochloride). Draw them in the space provided at the end of the book.

NOTE.—This test can be applied to dilute solutions of haemoglobin by acidifying with acetic acid, precipitating with freshly prepared tannic acid, and treating the dried precipitate on a slide with a trace of salt and glacial acetic acid as described above. Suspected blood stains on linen, instruments, etc., should be extracted with a little alkali, the solution evaporated to dryness and treated as above.

CHAPTER VIII.

THE CONSTITUENTS OF BILE.

For the following reactions use ox or sheep's gall.

190. Take the reaction with litmus paper: it is faintly alkaline.

191. To a small quantity add strong acetic acid, drop by drop. A precipitate is formed, insoluble in excess of acid. This precipitate consists of a nucleo-protein, together with a considerable amount of the bile salts and bile pigments.

NOTE.—The protein precipitated was formerly supposed to be mucin, owing to the insolubility in excess of acid. It has, however, been shown that it is a nucleo-protein, the insolubility in excess of acid depending on the presence of the bile salts (See Ex. 200). It is generally spoken of as "pseudo-mucin."

Human bile contains both mucin and nucleo-protein.

192. **Gmelin's test for bile pigments.** Take a few c.c. of fuming yellow nitric acid in a test tube and by means of a pipette, carefully place on the surface of this an equal amount of bile. Shake the tube very gently from side to side, and note the play of colours in the bile as it becomes oxidised by the acid. Proceeding from acid to bile the colours are yellow, red, violet, blue, and green.

NOTES.—This test can be modified in many ways.

1. Add a drop of yellow nitric acid to a thin film of bile on a

white porcelain plate. The drop of acid becomes surrounded by rings of the various colours.

2. Filter some diluted bile repeatedly through an ordinary filter paper, and then place a drop of fuming nitric acid on the paper. The play of colours is usually well seen.

193. **Modified Huppert's test for bile pigments.** To about 50 c.c. of diluted bile add an excess of baryta-mixture. Stir well, heat, and allow to stand for a short time. The precipitate, containing an insoluble barium compound of bilirubin, coheres together. Remove the main mass of the fluid by means of a pipette, and then filter. Open the filter paper on a tile and scrape the precipitate off the paper. Place it in a test-tube, add about 4 c.c. of strong alcohol, two drops of strong sulphuric acid, two drops of a 5 per cent. solution of potassium chlorate, and boil for a minute. Allow the precipitate of barium sulphate to settle. The supernatant alcohol is coloured a greenish-blue.

NOTES—1. If the precipitate obtained by the baryta-mixture is very slight, a small amount of sodium phosphate solution should be added to increase the bulk of the precipitate.

2. This test is especially valuable for detecting the presence of bile pigments in urine.

194. **Pettenkofer's test for bile salts.** To 5 c.c. of bile that has been diluted ten times add a small particle of cane-sugar and shake or warm till this has completely dissolved. To the cooled solution add 5 c.c. of concentrated sulphuric acid, inclining the test tube so that the acid settles to the bottom. Gently shake the test tube from side to side. As the fluids gradually mix a deep purple colour develops.

NOTES—1. This reaction depends on the production of furfurol from the cane-sugar by the strong acid (See Ex. 73).

2. If too much cane-sugar be taken the fluid will turn brown or black, owing to the charring produced.

3. Proteins give a very similar reaction with furfurol in the presence of strong acids. Proteins also tend to give a brown char with sulphuric acid. For these reasons it is advisable to remove the proteins from solution before attempting the test.

4. The purple colour obtained is only stable in the presence of strong sulphuric acid. It disappears on dilution with water.

5. If a small portion of the coloured fluid be diluted with 50 per cent. sulphuric acid and examined with the spectroscope, two absorption bands will be seen, one between the lines C and D, nearer the latter; the other in the green, overlapping E and b.

195. **Hay's test for bile salts.** To 50 c.c. of water in a beaker add ten drops of bile, stir well with a rod, and then allow the fluid to come to rest. Sprinkle some flowers of sulphur on the surface and note that they fall through the liquid to the bottom of the beaker. Repeat the test in a beaker of water without the addition of bile, noting that the particles remain on the surface.

NOTES—1. This test for bile salts depends on the remarkable property that they possess of lowering the surface tension of water, thus enabling the particles of sulphur to sink through the fluid.

2. The test is of great value for the detection of bile salts in urine. Pettenkofer's test is rarely obtained with certainty in urine owing to the presence of other chromogenic substances.

3. This property of bile salts is utilised by draughtsmen in preparing tracings on oiled paper, on which ink collects in drops, and does not spread well. If the paper be first treated with a little ox-gall and allowed to dry the difficulty is removed, owing to the reduction in surface tension.

4. A method for estimating bile salts in urine has been described by Grünbaum, depending on this property. The rate of escape of the urine from standard capillary tubes is noted, the rate increasing with the concentration of bile salts.

5. Owing to the low surface tension of alcohol, the presence of this must be excluded by evaporation before the test can be relied on.

6. Solutions of soaps also have rather a low surface tension, so that they must be excluded by the tests given in Ex. 111.

196. Mount a few crystals of **cholesterin** in water, examine under the microscope, and draw them. Note the rhombic plates, which are often incomplete at one corner. Irrigate the crystals with strong sulphuric acid : they turn red at the edges. Now add a drop of iodine solution : the crystals give a violet colour, changing to a green, blue, and finally a black.

197. ***Salkowski's* reaction for cholesterin.** Dissolve a little in a few c.c. of chloroform ; to the solution add an equal quantity of strong sulphuric acid and shake. The upper layer of chloroform becomes red, the layer of sulphuric acid yellow with a green fluorescence.

198. **Liebermann's reaction for cholesterin.** Dissolve a little cholesterin in 2 c.c. of chloroform, contained in a perfectly dry tube. Add ten drops of acetic anhydride, then two drops of strong sulphuric acid, and shake. The solution becomes coloured a deep blue.

199. Evaporate 20 c.c. of bile to complete dryness, finishing the evaporation on the water bath. Treat the residue with 20 c.c. of alcohol, and heat on the bath to boiling, rubbing the mixture well with a glass rod. Add a little more alcohol and filter. Evaporate the filtrate to dryness on the water bath, and then

extract it with 30 c.c. of hot water. Cool and filter again. A solution of the salts and pigments of bile is thus obtained free from proteins.

200. Acidify a portion of the solution thus obtained by means of two or three drops of strong acetic acid; note that the bile acids are not precipitated. To the acid solution thus obtained add an equal quantity of 1 per cent. solution of Witte's peptone. A white milkiness or a decided precipitate is produced, insoluble in excess of acid. (**Oliver's test for bile salts.**)

NOTE.—This test can also be applied to urine much more readily than Pettenkofer's test. The urine should be acidified with acetic acid, filtered till quite clear, and treated with an equal amount of a 1 per cent. solution of Witte's peptone.

201. Saturate the remainder of the solution obtained in Ex. 199 with ammonium sulphate. The bile salts and pigments are precipitated.

CHAPTER IX.

URINE AND ITS CHIEF CONSTITUENTS.

A. Urea—$CO(NH_2)_2$

202. To a watch-glass half full of distilled water add as much solid urea as will lie on a sixpenny-piece. Note the solubility of urea in water.

203. Place a drop of the urea solution on a slide, add a single drop of saturated solution of oxalic acid, mix by stirring with a needle or fine glass rod, cover with a slip and examine the crystals of *oxalate of urea* that separate out. They vary considerably, containing long, thin, flat crystals, often in bundles and rhombic prisms. Draw the crystals.

204. Dilute the urea solution with twice its volume of water. Place a drop on a slide, add a drop of pure nitric acid, cover with a slip, and examine the crystals of *urea nitrate* that separate out. They form octahedral, lozenge-shaped, or hexagonal plates, often striated and imbricated. Draw the crystals.

205. Powder two or three crystals of urea in a watch-glass: rub with a small amount of acetone and warm gently on a water bath. The urea dissolves. Allow most of the acetone to evaporate

away, and then place a drop of the remaining solution on a watch-glass. Urea crystallises out as the acetone passes off. Draw the crystals.

206. Repeat the above exercise, using strong alcohol instead of acetone. Draw the crystals of urea, which are usually very irregular.

207. Dilute the remainder of the aqueous solution left from Ex. 204 with an equal quantity of water, and to a portion of this in a test tube add some yellow nitric acid (or nitric acid to which a little potassium nitrite has been added). An effervescence and evolution of gas takes place.

$$CO(NH_2)_2 + 2\,HNO_2 = CO_2 + 2\,N_2 + 3\,H_2O.$$

NOTE.—All compounds containing the amido group (NH_2) react in a similar manner when treated with nitrous acid.

208. To another portion of the solution add sodium hypobromite. A marked effervescence and evolution of gas takes place.

$$CO(NH_2)_2 + 3\,NaBrO + 2NaHO = 3\,NaBr + Na_2CO_3 + 3H_2O + N_2.$$

209. To a few c.c. of saturated ammonium sulphate add sodium hypobromite, a marked effervescence and evolution of gas takes place.

$$(NH_4)_2SO_4 + 3\,NaBrO + 2\,NaHO = Na_2SO_4 + 5\,H_2O + 3\,NaBr + N_2.$$

NOTES—1. All ammonium salts and all compounds with the amido group give off nitrogen when treated with an alkaline solution of sodium hypobromite.

2. The sodium hypobromite is prepared as follows : dissolve 100

grams. of caustic soda in 250 c.c. of water. Cool, and slowly add 25 c.c. of bromine, cooling under the tap as the bromine is added. The reaction is as follows:

$$2NaHO + Br_2 = NaBrO + NaBr + H_2O.$$

It must be freshly prepared before use as it undergoes the following decomposition

$$3\ NaBrO = 2\ NaBr + NaBrO_3.$$

3. As a test for urea the reaction with hypobromite is only useful in a negative sense; that is to say, if an effervescence is not obtained urea is absent, but if an effervescence is obtained it does not necessarily follow that urea is present.

210. To some of the urea solution add a solution of mercuric nitrate. A white precipitate of mercuric oxide combined with urea nitrate takes place. To the mixture thus obtained add a saturated solution of sodium chloride, drop by drop. The precipitate dissolves, to reapppear on a further addition of mercuric nitrate.

NOTE.—The reaction is sometimes useful in detecting the presence of urea in solutions. Proteins give a precipitate with mercuric salts, which is soluble or insoluble in NaCl, depending on the nature of the protein. Therefore, to make the test more certain, proteins should be removed by the method given in Ex. 52. Since phosphates give a very similar reaction they must be removed by baryta mixture before testing for urea (See Ex. 234).

211. Treat a solution of urea with Millon's reagent, and heat. A white precipitate is formed, owing to the presence of mercuric nitrate in the reagent. There is also an evolution of gas due to the action of the nitrous acid on the urea.

212. Boil a dilute solution of urea with a little strong alkali for fifteen minutes. Cool, neutralise

with diluted sulphuric acid and test for urea by the addition of mercuric nitrate. No precipitate is obtained owing to the hydrolysis of the urea by the boiling alkali.

$$CO(NH_2)_2 + H_2O = CO_2 + 2NH_3.$$

213. Repeat the above exercise, using a few drops of strong sulphuric acid in place of the alkali, and then neutralising with sodium hydrate. The urea is hydrolysed as before.

NOTE.—In view of the ease with which the hydrolysis of urea is effected by boiling acids or alkalies, it is most important, when evaporating a solution to dryness as a preliminary to testing for urea, to neutralise the solution before evaporation.

214. Place a little urea in a dry test tube. Heat carefully over a flame, keeping the upper part of the tube cool. The urea melts and evolves ammonia, whilst a white sublimate condenses on the cooler parts of the tube. Cool the tube, add a little water and shake. Pour the solution into another tube and treat it with an equal bulk of sodium hydrate and a drop of copper sulphate. A pink colour is produced, due to the biuret formed from the urea. Biuret has the composition $NH_2 . CO . NH . CO . NH_2$.

B. Uric Acid.

215. Treat a small amount of uric acid with 10 c.c. of 2 per cent. sodium carbonate. Heat nearly to boiling and cool. Note that a considerable portion of the uric acid has dissolved in the form of a urate.

216. Filter the solution and treat a portion with a drop or two of strong hydrochloric acid and shake. A white crystalline precipitate of uric acid separates out, showing that uric acid is very insoluble in water. Allow the crystals to settle, remove a few by means of a pipette, and examine them microscopically. They usually form rhombic plates. Draw the crystals.

NOTE.—If the solution is very strong, the uric acid may separate out in an amorphous form. Should this be the case, make the solution alkaline and heat to dissolve. Whilst still hot add some HCl and allow the tube to cool slowly.

Uric acid can assume a great variety of crystalline forms, resembling dumb-bells, whetstones, butcher-trays, stars, and sheaves.

217. To another portion of the solution add two drops of ammonia and saturate with ammonium chloride. A white amorphous precipitate of ammonium urate is formed.

NOTE.—This is an important reaction of urates, since it is the basis of the method of estimating uric acid in urine, described in Ex. 250. It is especially reliable as a test, since no other organic substance, likely to be met with in physiological analysis, is precipitated by saturation with ammonium chloride. The murexide reaction (Ex. 219) can be applied to the precipitate obtained.

218. Treat a little uric acid with strong sulphuric acid: it readily dissolves.

219. Treat a little uric acid in a porcelain dish with two or three drops of strong nitric acid. Heat on the water-bath till every trace of nitric acid and water has been removed. A reddish deposit remains. Treat this with a dilute solution of ammonia (five drops of ammonia to about a test tube full of water). The residue turns to a violet colour. (**Murexide test.**)

NOTE.—This important test needs a certain amount of care. The heating must be performed on the water-bath and should be continued as long as is necessary to ensure the complete removal of every trace of nitric acid. If xanthin, and a few other substances found in urine and closely related to uric acid, be evaporated to dryness with nitric acid containing a little hydrochloric acid, a similar reaction is obtained.

220. Treat a very small amount of uric acid with a few c.c. of sodium carbonate. Pour the solution on to a filter paper moistened with silver nitrate. A black stain of reduced silver immediately results. **(Schiff's test.)**

NOTE.—This useful test cannot be applied in the presence of chlorides. It is important to note that the uric acid is dissolved in sodium carbonate, not the hydrate, as the latter gives a precipitate of the brown silver hydroxide, which completely obscures the reduction. An amount of sodium carbonate in excess of that required to dissolve the uric acid must be added, as the reduction only takes place in the alkaline condition.

221. Treat a little uric acid in a tube with 5 c.c. of Fehling's solution and boil for a considerable time. Note the peculiar reduction of the copper, and compare it with the reduction obtained with dextrose.

222. Dissolve a little uric acid in a small amount of sodium hydrate: place a drop on a slide and allow it to evaporate slowly. Sodium urate will crystallise out. Examine microscopically, and note the spheres covered with projecting spines or thin prisms. Make a drawing of these.

223. Dissolve some uric acid in sodium carbonate, add an excess of ammonia and treat with silver nitrate.

A white amorphous precipitate of a silver compound of uric acid is formed.

NOTE.—Xanthin, hypoxanthin and other substances in urine closely related to uric acid are similarly precipitated by ammoniacal silver nitrate.

C. Normal Urine.

224. Take the specific gravity of urine by means of a urinometer. Wipe the instrument clean, and float it in the centre of a cylinder containing the urine. Remove all bubbles, by means of filter paper or by placing a single drop of ether on the surface of the urine. Take care that the urinometer does not touch the sides of the vessel. Place the eye level with the surface of the fluid and read the division of the scale to which the latter reaches. Read the level of the true surface of the urine, not the top of the meniscus around the shaft of the urinometer.

Since the instrument is graduated for a temperature of 15°C., the urine should be allowed to cool to this temperature before an observation is taken.

The specific gravity of normal urine usually varies between 1015 and 1025. In the summer months it may be as high as 1035 even in health.

NOTE.—The total amount of solids in the urine can be thoroughly estimated from the specific gravity by multiplying the last two by 2.33. The result is in grams. per litre. Thus, if specific gravity is 1020, it contains $20 \times 2{\cdot}33 = 46{\cdot}6$ grams. per litre.

225. Test the reaction of fresh urine to litmus paper. It is usually acid: but it may react acid to

a blue paper and alkaline to a red paper (amphoteric reaction); or it may be alkaline (after a meal).

NOTE.—Urine is very liable to undergo alkaline fermentation, the urea being hydrolysed to ammonium carbonate by an organism (Micrococcus ureae). In such cases, if the litmus paper be gently warmed, the blue colour will disappear, showing that the alkalinity is due to ammonia or an ammonium salt.

For the method of estimating the acidity of urine, see Ex. 261.

226. Note the colour of normal urine and examine some in a beaker by the spectroscope. Note that there are no definite absorption bands, but a general absorption of the violet. Urochrome, the chief urinary pigment, yields no bands.

227. Saturate at least 200 c.c. of urine with ammonium sulphate. Filter off the precipitate and let it completely dry in the air. Extract it with a small amount of strong alcohol. A brownish solution containing urobilinogen is obtained. Treat this with a few drops of hydrochloric acid: the urobilinogen is converted to urobilin. Examine with the spectroscope, and note a single absorption band situated at the junction of the blue and the green. Its centre is about λ 490.

NOTE.—Free urobilin is not present in normal urine, but is found in the urine of patients suffering from certain febrile disorders.

228. Test for *chlorides* by adding to about 3 c.c. of urine a few drops of pure nitric acid and 3 c.c. of a 3 per cent. solution of silver nitrate. An abundant curdy precipitate of silver chloride appears at once. If the chlorides are diminished, the solution merely becomes milky or opalescent.

NOTE.—The chlorides of urine vary in amount: they are markedly decreased in febrile conditions. The quantitative estimation is described in Ex. 259.

229. To a test tube nearly full of urine add a little strong ammonia and boil. A white flaky precipitate of the *phosphates of calcium and magnesium* is formed. Filter off the precipitate, wash with water, and dissolve in 5 c.c. of dilute acetic acid. Divide the solution into two parts. To one part add a solution of potassium oxalate. A white precipitate is produced, showing the presence of *calcium* in the urine.

230. To the other portion of the solution add an equal bulk of strong nitric acid and about 5 c.c. of ammonium molybdate. Boil: a yellow crystalline precipitate is produced, showing the presence of *phosphates*.

NOTE.—Neutral urine is very apt to yield a precipitate of earthy phosphates on boiling, owing to the change of reaction due to the evolution of CO_2 (See notes to Ex. 8).

231. **To demonstrate the presence of acid-phosphates in urine.** Treat 5 c.c. of urine with an equal volume of 5 per cent. solution of barium chloride. Filter repeatedly through a small filter paper till the filtrate is clear. Treat the filtrate with a little baryta mixture and boil. Filter; dissolve the precipitate in nitric acid and boil the solution obtained with ammonium molybdate. The yellow precipitate shows the presence of acid phosphates (as NaH_2PO_4) in the urine.

NOTE.—Any alkaline phosphate, Na_2HPO_4, present in the urine is precipitated by $BaCl_2$ as $BaHPO_4$. The acid phosphates remain

in solution as $Ba(H_2PO_4)_2$. On the addition of the alkaline baryta mixture, the acid phosphate is converted to the insoluble alkaline phosphates of barium. If no precipitate is produced when the baryta-mixture is added, there are no acid phosphates present in the sample of urine.

Since the acidity of a sample of urine varies almost directly with the amount of acid phosphate present, as determined by the above method, it is generally held that the acidity of urine is mainly due to the presence of these acid phosphates.

232. Treat 10 c.c. of urine with a few drops of strong hydrochloric acid, and about 3 c.c. of a solution of barium chloride. A precipitate of barium *sulphate* is produced as an opaque milkiness. If the precipitate is thick the sulphates are in excess. (The hydrochloric acid is added to prevent the precipitation of phosphates.)

233. To urine add an equal bulk of baryta mixture (two parts of baryta water to one part of a 10 per cent. solution of barium nitrate). A precipitate is formed consisting of the phosphates and the ordinary *inorganic sulphates*. Filter till quite clear. To the filtrate add a third of its volume of strong hydrochloric acid, boil in a beaker for five minutes, and allow to stand. A faint white cloud of barium sulphate is formed indicating the presence of *ethereal sulphates* in the urine.

NOTES—1. The ethereal sulphates form soluble barium salts, but are hydrolysed to sulphuric acid by heating with an acid.

$$\begin{matrix} C_6H_5-O \\ \qquad\quad \diagdown \\ \qquad\quad SO_2 \\ \qquad\quad \diagup \\ HO \end{matrix} + H_2O = C_6H_5 . OH + H_2SO_4.$$

Phenol-sulphuric acid. Phenol.

The sulphuric acid thus formed is converted to barium sulphate by the excess of barium present.

2. The solution becomes very dark in colour on boiling with the strong acid, owing to its action on the aromatic chromogenic substances in the urine.

234. Treat 5 c.c. of urine with half its bulk of baryta mixture, and filter off the precipitate of sulphates and phosphates. Neutralise the filtrate with acetic acid and add a little mercuric nitrate. A white precipitate, soluble in sodium chloride, is obtained, indicating the presence of *urea*.

235. Evaporate about 50 c.c. of urine to complete dryness, finishing the evaporation on the water bath. Rub the residue with about 10 c.c. of acetone, and pour off the acetone into a clean evaporating basin. Place this on the water bath till half of the acetone has evaporated; remove it from the bath and pour a drop of the solution on to a glass slide. Crystals of urea separate out as silky needles as the acetone evaporates away. Examine them microscopically. Evaporate the remainder of the solution to dryness, dissolve the residue in a minimal amount of water, place a drop on a slide, add a drop of nitric acid, and examine microscopically the crystals of urea nitrate that form.

236. **To demonstrate the presence of Uric Acid in urine.** Treat 50 c.c. of urine with two drops of ammonia and then stir with powdered ammonium chloride till the solution is saturated, avoiding an excess of the salt. Note the gelatinous precipitate of ammonium urate. Filter: scrape the precipitate off the paper and transfer it to an evaporating dish. Add three or four drops of strong nitric acid and place the dish on the water bath till a pink, dry residue is obtained. Treat this with a little dilute

ammonia: the purple colour produced indicates the presence of urates in urine. (See Exs. 217 and 219.)

237. Urine has been treated with about one-fiftieth its bulk of strong hydrochloric acid, and allowed to stand from twelve to twenty-four hours. Note the brown crystals of *uric acid* that have formed on the sides of the vessel. Examine them microscopically: they form very irregular crystals, usually arranged in sheaves. Draw the crystals.

NOTE.—The chief pigment that associates itself with uric acid and urates is known as uroerythrin.

238. Boil 3 c.c. of urine with 3 c.c. of Fehling's solution for two minutes, and allow the tube to cool. A very slight reduction is obtained, due partly to urates (See Ex. 221), and kreatinin, and partly to the very small amount of dextrose that is present in normal urine.

239. To filtered urine apply the various protein tests (see below). All proteins are absent.

D. Certain Constituents of Abnormal Urine.

Albumin. Urine may contain albumins or globulins, the condition being known as "albuminuria." Albumins are more common than globulins, and the term "albumin" is usually held to include globulins also.

240. Take 10 c.c. of urine (filtered, if necessary, till quite clear) in a test tube. If it is neutral or alkaline, render it *faintly* acid with acetic acid and boil. Test the reaction whilst boiling, and, if necessary, add a drop or two of dilute acetic acid

till the reaction is again faintly acid. A precipitate or turbidity may be due to coagulated albumin or to earthy phosphates. Add a drop of nitric acid. Any turbidity remaining is due to the presence of albumin.

NOTE.—The amount of albumin present can be roughly estimated from the density of the coagulum produced.

241. Place about 3 c.c. of pure nitric acid in a narrow test tube. Float about 3 c.c. of filtered urine on the surface of this, using a pipette to avoid mixing. A white ring at the junction of the fluids indicates the presence of albumin. (**Heller's test.**)

NOTES—1. The white ring is due to the formation of meta-protein, and by the action of the acid on the albumin, and the insolubility of the meta-protein in the strong nitric acid. (See Exs. 1, 12, and 27.)

2. A coloured ring is often produced owing to the oxidation of certain urinary chromogens.

3. In very concentrated urine, a white ring of urea nitrate may form.

4. If the urine is very rich in urates, a precipitate of uric acid may form at the junction of the fluids. Urea and uric acid are distinguished from albumin by the previous dilution of the urine with two or three volumes of water.

5. The presence of resinous substances in the urine of patients who have been treated with balsams leads to the development of a white ring or cloud that disappears on treatment with alcohol.

6. Urine rich in albumose may give a white cloud that disappears on warming.

242. Render the urine faintly acid with acetic acid, and repeat the above test, using Spiegler's reagent instead of nitric acid. A white ring indicates the presence of albumin. (**Spiegler's test.**)

NOTES—1. Spiegler's reagent consists of mercuric chloride 8 grams., tartaric acid 4 grams., glycerine 20 grams., NaCl 10 grams., distilled water 200 c.c.

2. The reaction is also given by albumoses and peptones.

243. **Albumose and peptone.**

Remove any albumin that may be present by heat-coagulation, and to the filtrate apply Spiegler's reaction. A white ring indicates the presence of albumose or peptone.

244. Remove albumin as before, add an equal volume of saturated sodium chloride and then strong acetic acid, drop by drop, till no further cloud is formed. If this cloud disappears on warming and reappears on cooling, albumoses are present.

Dextrose.

245. Remove any albumin that may be present by heat-coagulation. Boil 3 c.c. of the filtrate with 3 c.c. of Fehling's solution and compare the reduction with that obtained in Ex. 238. If more than 1 per cent. of sugar is present a definite yellow or red precipitate is produced. If less than 0·8 per cent. is present the reduction is best seen as the fluid cools.

246. In a test tube place solid phenyl-hydrazine-hydrochloride to a depth of half-an-inch, twice this amount of solid sodium acetate and 10 c.c. of the urine that has been freed from albumin. Heat till the solids have dissolved, mix, and leave in a beaker of boiling water for half-an-hour. Allow the tube to cool slowly, and examine the deposit, if any, for the characteristic crystals of phenyl-glucosazone (See Ex. 66.)

NOTE.—This test is very useful in distinguishing dextrose from lactose (See Ex. 68) and from glycuronic acid, both of which give osazones of a different crystalline form.

247. Fill a test tube with the urine and add a piece of washed brewer's yeast about the size of a pea. Shake well and invert, placing the open end under mercury contained in a small dish. Clamp the tube in position, and allow it to stand for at least eighteen hours in a warm place. If dextrose is present in the urine there is an accumulation of gas (CO_2) at the top of the tube.

NOTES—1. This is an excellent method of confirming the presence of dextrose in diabetic urine.

2. If the specific gravity of the urine be taken before, and after complete fermentation with yeast, the lowering of the specific gravity is a rough index of the amount of sugar present.

The Blood-Pigments.

If only a small amount of blood or blood-pigment be present in urine, this has a peculiar reddish-brown opaque appearance, to which the term "smoky" is sometimes given.

If a larger amount be present the urine is red.

If blood corpuscles are present the condition is spoken of as "haematuria"; if only haemoglobin, the condition is that of "haemoglobinuria." Methaemoglobin is nearly always present in haemoglobinuria, and contributes largely to the smoky appearance.

248. Examine the urine in a beaker with a spectroscope. The bands of oxyhaemoglobin or of methaemoglobin may be seen after suitable dilution.

249. If they cannot be recognised owing to the small amount present, boil the urine with a few drops of strong ammonia, allow it to cool somewhat,

and add a few drops of ammonium sulphide, and examine for the bands of haemochromogen. (See Ex. 186.)

250. Boil 10 c.c. of the urine with a little 40 per cent. sodium hydrate, and allow the tube to stand for a while. A red deposit indicates the presence of blood-pigment in the urine.

NOTE.—The soda converts the haemoglobin to alkaline haematin, which adheres to the precipitate of earthy phosphates that is also formed.

Bile.

251. Apply Gmelin's test for bile pigments (Ex. 192). Only the green stage is conclusive, the other colours being often produced by the action of the nitric acid on certain chromogens in the urine.

252. Apply Huppert's test for bile pigments to 50 c.c. of the urine (Ex. 193).

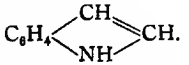
253. Apply Hay's test for bile salts (Ex. 195).

254. Apply Oliver's test for bile salts (Ex. 200).

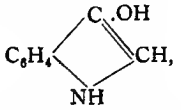
Indican.

255. Treat 5 c.c. of horse's urine with 5 c.c. of concentrated hydrochloric acid and about 2 c.c. of chloroform. Add a single drop of 3 per cent. potassium chlorate and shake. Allow the chloroform to settle and examine its colour. If it is blue, indican is present. If not, add another drop of the chlorate and shake again. If no blue colour is found in the chloroform, indican is absent.

NOTE.—Indican is the potassium salt of indoxyl-sulphuric acid. It arises primarily from the bacterial decomposition of tryptophane in the intestine. Tryptophane or indol-amido-propionic acid (Ex. 3) is converted by the bacteria to indol.



This is absorbed and oxidised to indoxyl.



which is excreted as indican. The above test depends on the hydrolysis of the indican to indoxyl and sulphuric acid by the HCl employed, and the subsequent oxidation of the indoxyl to indigo-blue which is soluble in chloroform. If too much of the oxidising reagent be employed the indigo-blue is oxidised to a colourless compound.

256. **Urinary deposits** (inorganic).

I. In *acid urine:*

Uric acid: deeply tinged with a bright red pigment. For crystalline form see Ex. 216.

Urates: pinkish, soluble on warming, generally amorphous, but may form star-shaped clusters with projecting spines.

Calcium oxalate: octahedra, with an envelope-like appearance (squares crossed by two diagonals); also in dumb-bells.

Calcium hydrogen phosphate (stellar phosphates): in rosettes of prisms, and in dumb-bells (rather rare).

II. In *alkaline urine:*

Ammonium urate: spheres, with or without projections.

Ammonium magnesium phosphate (triple phosphate): prisms (coffin-lids) or feathery stars.

Calcium phosphate: amorphous.

Calcium hydrogen phosphate (stellar phosphates).

Calcium carbonate: dumb-bells or spheres with radiating structure.

If you have an opportunity of seeing any of these sediments make a drawing of them.

CHAPTER X.

THE QUANTITATIVE ANALYSIS OF URINE.

257. A. The estimation of urea by the hypobromite method.

Principle. Urine is treated with an alkaline solution of sodium hypobromite and the amount of urea calculated from the volume of nitrogen evolved.

The reaction that takes place is as follows:—

$$CO(NH_2)_2 + 3\ NaBrO + 2\ NaHO = N_2 + 3\ NaBr + Na_2CO_3 + 3\ H_2O.$$

Hence 60 grams. urea evolve 28 grams. N.

$$= 2 \times 11{\cdot}2 \text{ litres},$$

and 1 gram. urea evolves 373 c.c. N.

Practically it is found that only 357 c.c. are evolved, the other 4·4 per cent. of the nitrogen being converted to nitrates, cyanates, etc. On the other hand, there are other substances in the urine that evolve nitrogen, such as ammonia, kreatinine and uric acid. Therefore the method only gives approximate results. It is, however, of great use clinically, as the amount of nitrogen evolved is a rough measure of the metabolism of the subject.

Apparatus. A 50 c.c. burette is held by a clamp in a tall cylinder of water. The upper end of the

burette is closed by a tightly-fitting rubber stopper, which is pierced by one limb of a glass T-piece. The upper limb of the T-piece is fitted with a short length of pressure-tubing carrying a screw-clamp. The side limb of the T-piece is connected by about two feet of small rubber tubing to a glass tube piercing the well-fitting rubber stopper of a wide-mouthed bottle of about 60 c.c. capacity. This bottle is placed in a jar of water, supported at such a height that the burette can be lifted nearly out of the tall cylinder without stretching the rubber connexion. A small glass bottle or short tube of 10 to 15 c.c. capacity is also required. For the method of preparing the hypobromite solution see Ex. 209.

Method of Analysis. Place 25 c.c. of freshly-prepared hypobromite solution in the larger bottle. Put 5 c.c. of urine, accurately measured, in the small bottle, and place this inside the other by means of a pair of forceps, taking great care not to upset any urine into the hypobromite. Fit the rubber cork tightly into the bottle and place this in the jar of water to cool. See that the burette is as low as possible, that the cylinder has sufficient water in it to reach the zero graduation of the burette, and that the screw clamp is open. Leave the apparatus for about a minute to cool to the temperature of the water; clamp the burette in such a position that the water is below the zero mark, and then screw the clamp on the rubber tubing as tight as possible. Note down on paper the level of the water in the tube, placing the

eye level with the meniscus. Take the bottle out of the jar, and gently tilt it so that the urine flows into the hypobromite.

Gently shake the bottle from side to side, keeping the bottle upright to prevent the froth from being forced up into the tube. Tilt the bottle again and repeat the process till the urine and hypobromite are thoroughly mixed. Place the bottle back in the jar of water for about a minute to cool. Raise the burette till the level of water in the tube is the same as that outside, the gas being thus under atmospheric pressure. Read the level of the meniscus as before: the difference in the two readings is the volume of nitrogen evolved. Ascertain the temperature of the water and the barometric pressure.

Calculation of results.

Let the temperature be $t°$ C., the tension of aqueous vapour at this temperature be T mm. (See Appendix), and the barometric pressure be B mm. of mercury. Let v be the volume of nitrogen measured under the conditions: at 0° C. and 760 mm. this will become

$$\frac{v \times 273 \times (B-T)}{(273+t) \times 760} = v'$$

Now 357 c.c. of N are evolved from 1 gram. of urea.

$\therefore$ v' c.c. are evolved from $\frac{v'}{357}$ gram. of urea.

$\therefore$ 5 c.c. urine contain $\frac{v'}{357}$ gram. urea.

and 100 c.c. urine contain $\frac{20v'}{357}$ gram. urea.

NOTE.—Performing these two calculations in one operation we obtain for the percentage of urea

$$\frac{v \times (B-T) \times 273 \times 20}{(273+t) \times 760 \times 357} = \frac{v \times (B-T)}{(273+t)} \times \cdot 0201.$$

The average amount of urea excreted in a day is about thirty grams. Since the amount of urine is about 1500 c.c., the percentage of urea is normally about 2.

258. **B. The estimation of uric acid** (Hopkins' method).

Principle. The urine is saturated with ammonium chloride, which completely precipitates the uric acid as ammonium urate. This is filtered off, decomposed with HCl, and the uric acid allowed to crystallise out. The uric acid is dissolved in sodium carbonate, treated with sulphuric acid, and titrated with a standard solution of potassium permanganate.

Reagents required.

(i) Pure ammonium chloride.

(ii) A filtered saturated solution of ammonium chloride contained in a wash bottle.

(iii) Concentrated HCl.

(iv) Concentrated H_2SO_4.

(v) Sodium carbonate solution, 2 per cent.

(vi) Twentieth normal solution of potassium permanganate, made by dissolving 1·581 grams. of the pure salt in water and diluting to one litre.

Method of Analysis. Into a beaker, of about 150 c.c. capacity place 28 grams. of ammonium chloride (approximately weighed). Measure into this 100 c.c. of the urine, add about five drops of strong ammonia and stir briskly till all the salt has dissolved. Note the pinkish precipitate of ammonium urate that floats to the surface. Stir briskly at intervals till the precipitate completely subsides. When the supernatant fluid is clear, pour this on to a small filter paper (9 cm. diam.) in a funnel of such size that only a small margin of glass projects above the edge of the folded paper. Do not transfer the precipitate to the paper until the greater part of the fluid has been filtered. Finally, transfer the whole of the precipitate to the paper, washing out the last traces by means of a saturated solution of ammonium chloride in a wash bottle. When the fluid has completely drained away, wash the precipitate on the paper once with the saturated solution.

Have ready a wash-bottle containing boiling distilled water and fitted with a *fine* jet. When the last washings have run through the filter paper, hold the funnel horizontally over a clean beaker, and wash the precipitate off the paper into the beaker with a jet of the hot water, using as small an amount of water as possible. When the bulk of the precipitate has been removed, take the paper carefully out of the funnel, unfold it, and remove the last traces of the urate from its folds. Not more than 30 c.c. of water should be employed: if much more than this has been used, the

solution must be concentrated on a water bath till about 30 c.c. remain.

Add six drops of strong hydrochloric acid to the solution, heat the beaker over a small flame till it just boils, label it, cover with a filter paper, and leave over night for the uric acid to crystallise out.

Filter the uric acid off through a very small paper (5 cm. diam.), collecting the filtrate in a graduated cylinder: note the exact amount of this filtrate, which is a saturated solution of uric acid in dilute HCl and ammonium chloride (See below). Wash the uric acid that remains in a beaker with a little cold water and pour this on to the filter: repeat this once or twice more to remove chlorides. Now wash the uric acid on the filter back into the beaker with a fine jet of hot water; add 5 c.c. of two per cent. Na_2CO_3, and about 50 c.c. of water and heat to boiling to dissolve the uric acid. Transfer the solution quantitatively to a 250 c.c. Erlenmeyer flask, that has been previously marked with a label at 100 c.c. Rinse the beaker out with water, and add this to the flask till the contents reach the 100 c.c. mark. Cool the mixture under the tap to room temperature.

Have the standard permanganate ready in a burette provided with a glass tap (permanganate attacks rubber tubing and so alters in concentration). Read the level of the meniscus by holding a lighted match behind it. To the cooled solution of sodium urate add 20 c.c. of concentrated sulphuric acid, shake and titrate at once without cooling.

During the titration the fluid in the flask must be kept in vigorous movement. Each drop of the permanganate is at first discoloured almost immediately, before it has had time to diffuse through the liquid and impart to it a pink tinge. The first instantaneous appearance of a diffuse flush through the solution marks the end point of the titration. The colour very rapidly disappears, but it will now be found that if another drop of permanganate be added, it has time to diffuse through the liquid, before it, in its turn, is decolourised.

Calculation of results.

1 c.c. of $\frac{N}{20}$ permanganate = ·00375 gram. uric acid.

Add ·001 gram. for every 15 c.c. of the mother liquor from which the crystals separated.

Example. Amount of urine in twenty-four hours was 1365 c.c. The uric acid from 100 c.c. of this required 12·4 c.c. of permanganate: the mother liquors from the same being 33 c.c.

$$12{\cdot}4 \times {\cdot}00375 = {\cdot}0465 \text{ gram.}$$
$$001 \times \tfrac{33}{15} = {\cdot}0021 \text{ gram.}$$
$$\underline{\quad\quad\quad\quad}$$
$$0486 \text{ gram. per cent.}$$

Amount of uric acid in twenty-four hours was therefore ·0486 × 13·65 = ·66 gram.

259. **C. The estimation of chlorides in urine** (Volhard's method).

Principle. The chlorides are precipitated from urine by a known excess of standard solution of silver nitrate in the presence of nitric acid. The excess of

silver is estimated in an aliquot portion of the filtrate by titration with the solution of potassium sulphocyanide, that has been previously standardised against the silver solution, a ferric salt being used as an indicator in both titrations.

Reagents required.

(i) Standard silver nitrate solution prepared by dissolving 29·063 grams. of pure fused silver nitrate in distilled water and filling up accurately to one litre. The solution should be kept in the dark.

1 c.c. corresponds to ·01 gram. NaCl (·00606 gram. Cl).

(ii) Solution of potassium sulphocyanide made by dissolving 8 grams. of the salt in a litre of distilled water.

(iii) Pure nitric acid, quite free from chlorine.

(iv) A concentrated solution of iron alum.

Standardisation of the Sulphocyanide. In a beaker place 10 c.c. of the silver nitrate, accurately measured: add 5 c.c. of pure nitric acid, 5 c.c. of iron alum and 80 c.c. of distilled water. Titrate the whole with the sulphocyanide from a burette until a faint permanent red tinge is obtained. Note the amount required for the 10 c.c. of silver nitrate.

Method of Analysis. In a 100 c.c. cylinder or measuring flask place 10 c.c. of urine, accurately measured by a pipette; 20 c.c. of the standard silver solution, also accurately measured; about 4 c.c. of pure nitric acid, and 5 c.c. of the iron alum. Add

distilled water till the 100 c.c. mark is just reached, and mix thoroughly by pouring into a beaker and stirring well. Filter off the precipitated silver chloride through a dry paper into a dry vessel. Of the filtrate take 50 c.c., accurately measured, and titrate it with the potassium sulphocyanide solution till a faint permanent red tinge is obtained.

Calculation of results. Example:

It was found that

$$19{\cdot}6 \text{ c.c. KCNS} = 10 \text{ c.c. } AgNO_3.$$

$$\therefore 1 \text{ c.c. KCNS} = \frac{10}{19{\cdot}6} \text{ c.c. } AgNO_3.$$

10 c.c. of urine with 20 c.c. of silver were made up to 100 c.c. with water, HNO_3 and the iron salt.

The silver is only partially used in precipitating the whole of the chlorides in the 10 c.c., a certain amount of the silver being left in excess. The amount left in 50 c.c. corresponded to 11·6 c.c. of KCNS. So the amount in 100 c.c. corresponded to

$$23{\cdot}2 \text{ c.c. of KCNS} = \frac{23{\cdot}2 \times 10}{19{\cdot}6} \text{ c.c. of } AgNO_3.$$

$$= 11{\cdot}8 \text{ c.c. of } AgNO_3.$$

$$\therefore (20 - 11{\cdot}8) \text{ c.c.} = 8{\cdot}2 \text{ c.c. of } AgNO_3$$

were precipitated by the chlorides in 10 c.c. of urine.

Now 1 c.c. of $AgNO_3$ = ·01 gram. of NaCl.

$\therefore$ 10 c.c. of urine contains ·082 gram. NaCl.

$\therefore$ 100 c.c. of „ „ ·82 gram. NaCl.

NOTE.—It is very important to remember to add the nitric acid, It renders the silver chloride insoluble and prevents the precipitation of the silver compounds of the purine bases in those cases in which the urine is alkaline.

260. **D. The estimation of phosphates in urine.**

Principle. Urine is heated to boiling point, and titrated whilst hot with a standard solution of uranium acetate, which gives a precipitate $(UrO_2)HPO_4$ with phosphates in acetic acid solution. Cochineal tincture is used to indicate by a change in colour when the uranium is in excess.

Reagents required.

1. A solution containing 100 grams. of sodium acetate and 100 c.c. of strong acetic acid to a litre of distilled water.

2. Cochineal tincture, prepared by extracting the insects with 30 per cent. alcohol and filtering after two days.

3. A standard solution of sodium phosphate. Dissolve twelve grams. of pure sodium phosphate in a litre of distilled water. Take 50 c.c. of the filtered solution and evaporate it to dryness in a weighed dish or crucible on a water bath. When dry, raise the temperature to about 130° C., and leave for some hours. Allow the dish to cool in a dessicator and weigh. Let x be the weight of the pyrophosphate obtained. Then to every 100 c.c. of the remaining solution add from a burette $\frac{(x-\cdot3746)}{\cdot003746}$ c.c. of distilled water. A solution is thus obtained of such a strength that 50 c.c. = ·1 gram. P_2O_5.

4. Standard solution of uranium acetate. Dissolve by the aid of heat 35 grams. of uranium acetate in a

litre of water. Allow the solution to cool and then filter. Standardise the solution as follows: In a beaker place 50 c.c. of the phosphate solution, add 5 c.c. of the sodium acetate solution and a few drops of the cochineal tincture. Bring the solution to the boiling point, remove the flame and titrate with the uranium acetate solution from a burette till the red tinge just changes to a green, heating the mixture to boiling before the last few drops are added. Suppose x c.c. of the uranium are necessary: then to each 100 c.c. of the remaining portion add $\frac{(20-x)\times 100}{x}$ c.c. of distilled water. A standard solution of uranium acetate is thus obtained, of which 1 c.c. = ·005 gram. P_2O_5.

Method of Analysis. In a beaker of about 100 c.c. capacity place 50 c.c. urine, add 5 c.c. of the sodium acetate solution and a few drops of the cochineal tincture. Have a burette ready containing the standardised uranium acetate solution. Heat the urine to boiling point, remove the flame and run in the uranium acetate as long as a precipitate is formed. Heat the mixture again just to boiling point, and cautiously add uranium, drop by drop, till the red colour is converted to a green.

Calculation of results.

1 c.c. of the uranium acetate = ·005 gram. P_2O_5.

Thus if 50 c.c. of urine require 31 c.c. uranium, the percentage of P_2O_5 is $2\times 31\times ·005 = ·31$ gram.

261. E. The estimation of the acidity of urine.

Solutions required.

(i) A decinormal solution of sodium or potassium hydrate. This can be purchased or can be prepared according to the directions given in Sutton's *Volumetric Analysis.*

(ii) A 1 per cent. alcoholic solution of phenol phthalein.

Method of analysis. Measure 10 c.c. of urine into a flask or beaker. Add four drops of the phenol phthalein, and 90 c.c. of distilled water.

Titrate with the standard alkali till a faint tinge of red appears in the mixture (See Note below).

Calculation of results. Express the acidity in terms of $\frac{N}{10}$ KHO. Thus if 3·1 c.c. of $\frac{N}{10}$ KHO are required for 10 c.c. urine, the acidity of the same is equivalent to 31 c.c. $\frac{N}{10}$ KHO per cent.

NOTE.—The method only gives approximate results owing to the difficulty in determining the end-point. Since the point varies with the amount of indicator used, this must be kept constant. Each individual worker should choose the end-point that he can most readily determine, and always proceed to this point. In this way reliable information can be obtained concerning the relative acidity of various samples of urine.

262. F. The estimation of the total nitrogen by Kjeldahl's method.

Principle. The nitrogenous compounds in 5 c.c. of urine are converted to ammonium sulphate by boiling

sulphuric acid, copper sulphate and potassium sulphate being added to aid the oxidation. The mixture is diluted with water, made alkaline by the addition of sodium hydrate and the ammonia distilled into a measured amount of standard acid. The amount of this neutralised by the ammonia is found by subsequent titration with standard alkali. Knowing the amount of ammonia formed from 5 c.c. of urine, the percentage of nitrogen can be readily calculated.

Reagents required.

(i.) Pure concentrated sulphuric acid, free from nitrogen.
(ii.) Crystalline potassium sulphate.
(iii.) Crystalline copper sulphate.
(iv.) Pure caustic soda in sticks.
(v.) Quarter normal sulphuric acid.
(vi.) Eighth normal sodium hydrate.
(vii.) A 1 per cent. alcoholic solution of methyl orange; or an aqueous solution of neutral litmus.

Method of Analysis.

Into a clean, dry, round-bottomed flask of Jena glass with a welted neck, of about 450 c.c. capacity (A, fig. 2), place 3 grams. of potassium sulphate, and 1 gram. of copper sulphate. Add 5 c.c. of the urine, accurately measured with a standard pipette, and 5 c.c. of the pure sulphuric acid. Place a funnel in the neck of the flask, to prevent any loss by spurting, and heat to boiling on a sand bath. The fluid should be kept *gently* boiling for about an hour, or until it becomes perfectly clear. Allow the flask to cool. Measure

5 c.c. of strong sulphuric acid into a beaker, dilute with water and ascertain what length of stick soda is required to neutralise the acid and make the reaction alkaline (about 3 inches are usually sufficient).

Into an Erlenmeyer flask (C) of about 400 c.c. capacity, place 25 c.c. (accurately measured) of a quarter-normal solution of sulphuric acid.

Fig. 2.—*Apparatus for Kjeldahl's method.*
For description see text.

When the flask A is cool add 200 c.c. of distilled water, and cool under the tap. Add the length of stick soda that has been found necessary and immediately fit the glass tube B into the neck of the flask by means of a well-fitting rubber stopper. The other end of B dips just below the surface of the standard acid in C. This is placed on an adjustable stand, in order that, as the distillation proceeds, the flask can be lowered so that B only just dips below the surface of the acid. This is necessary to prevent any of the acid being sucked back into A.

Now heat the mixture in A to boiling, resting the flask on a tripod covered with asbestos gauze, and supporting the neck by a metal clamp. As the alkali neutralises the acid, the copper sulphate is first converted to the blue hydrate, and then to the black oxide. Allow the fluid to boil till at least half the total volume of fluid has distilled over, lowering C from time to time as directed above. Finally lower C so that the tube no longer dips below the surface of the distillate and continue the boiling for another minute or two to wash down any of the standard acid that may have been sucked up into the tube or the bulb on it. Wash down the exterior of the tube with a jet of distilled water, allowing the washings to run into C.

To the fluid thus obtained add one drop of the methyl orange (or two or three drops of the litmus solution) and titrate with eighth normal alkali from a burette.

Calculation of results. Example:

17·8 c.c. of $\frac{N}{8}$NaOH were found necessary.

$\therefore \left(25 - \frac{17{\cdot}8}{2}\right) = 16{\cdot}1$ c.c. of $\frac{N}{4}$ acid were neutralised by the ammonia from 5 c.c. of urine.

100 c.c. of $\frac{N}{4}$ acid are equivalent to $\frac{1{\cdot}4}{4}$ grams. of nitrogen.

16·1 c.c. of $\frac{N}{4}$ acid are equivalent to

$$\frac{1{\cdot}4 \times 16{\cdot}1}{4 \times 100} = {\cdot}5563 \text{ gram. of nitrogen.}$$

$\therefore$ nitrogen in 100 c.c. $= {\cdot}5635 \times 20 = 1{\cdot}127$ grams.

CHAPTER XI.

MISCELLANEOUS EXERCISES.

263. **A. To demonstrate the presence of sugar in blood.** To 20 c.c. of blood in a porcelain dish add about 80 c.c. of tap water and boil. Whilst boiling add *dilute* acetic acid, drop by drop, until the reaction is faintly acid. Filter through a pleated paper, reserving the coagulum for the next excercise. The filtrate should be colourless and quite clear. If it is not so, add dilute acid or dilute alkali till the reaction is neutral; boil again and filter. If necessary concentrate the filtrate on the water bath and apply Fehling's test.

264. **B. To demonstrate the presence of iron in blood.** Take the coagulum obtained in the previous exercise and press it between two or three filter papers to remove the excess of moisture. Place a piece, about the size of a large bean, in a crucible supported by a pipe-clay triangle on a high tripod or metal ring. Add about a thimble-full of fusion mixture and heat by the Bunsen flame, at first cautiously and then strongly; touch the melt with the end of a glass rod from time to time to ensure thorough mixture. It is advantageous to complete the fusion process by means of a Fletcher's burner. Allow to cool; place the

crucible in a beaker and add fuming hydrochloric acid diluted with an equal volume of water until the reaction is acid. The iron is now in the form of ferric chloride and its presence can be detected either by obtaining a blue colour with potassium ferrocyanide, or a red colour with potassium sulphocyanide.

265. **C. To prepare a solution of myosin from meat.** Have the meat minced. Pound a watch-glass full with water in a mortar to remove the salts, extractives, soluble proteins, pigments, etc. Strain through muslin. Grind the residue with sand; and then pound with about a test-tube full of a 10 per cent. solution of ammonium chloride. The extraction should be allowed to go on for as long as possible, the grinding and pounding being repeated at intervals. Strain through muslin and filter through a large pleated paper moistened with ammonium chloride. The filtrate, which comes through very slowly, is a crude solution of myosin. If it be diluted about six times with distilled water, a considerable proportion of it is precipitated. The precipitate can be collected by decantation or otherwise and redissolved in salt solution. (See Ex. 35.)

266. **D. To prepare a solution of maltose from flour.** Make some of the flour into a stiff dough. Place a piece about the size of a walnut in muslin, and repeatedly knead it in water contained in a porcelain dish. The starch grains come through the muslin together with the soluble proteins. Pour the suspension into a beaker and let it stand till the

greater bulk of the starch has settled to the bottom. Carefully pour off the fluid. The starch must now be gelatinised. Boil about 50 c.c. of water in a beaker. When boiling add the starch grains which have been shaken up with about 5 c.c. of cold water. Boil for about a minute. Allow the starch paste to cool to about 40°C. Add a few c.c. of your saliva and digest on the water bath at 40°C, adding a few drops of a saturated solution of common salt (See Ex. 143). The best results are obtained after a prolonged digestion; the "achromic point" at least must be passed (See Ex. 142). The solution thus obtained contains maltose together with the saliva, sodium chloride, and achroo-dextrins. If it be desired to isolate the maltose, the solution must be evaporated to dryness (finishing the process on the water bath) and extracted with strong alcohol. The alcoholic solution of maltose is evaporated to dryness on the water bath, and the maltose dissolved in water. Crystals of maltosazone can be prepared from such a solution by the method given in Ex. 66.

267. **E. To prepare a solution of dextrose from flour.** Prepare starch paste by the following method. To the paste add about 5 c.c. of strong hydrochloric acid and boil for at least ten minutes, replacing the water as it evaporates away. The starch is thus hydrolysed completely to dextrose. Carefully neutralise with sodium hydrate, using at first 40 per cent. (probably more than 4 c.c. will be required) and finishing with 5 per cent. Care should be taken not to make the reaction

alkaline, as this destroys dextrose (See Ex. 61). The solution now contains dextrose and sodium chloride. Evaporate to dryness; extract the residue with strong alcohol, which dissolves the sugar only. Filter and evaporate the alcoholic solution to dryness. Dissolve the residue in water. If it be desired to prepare a specimen of dextrosazone, there is no need to remove the sodium chloride after neutralisation.

268. **F. A solution of sodium urate and urea is provided. To prepare crystals of uric acid and of urea.**

Heat nearly a test tube full of the solution to boiling point and add strong hydrochloric acid till the reaction is distinctly acid. Allow the tube to cool slowly; the uric acid crystals separate out. Cool thoroughly under the tap. Filter off the uric acid. Neutralise the filtrate with sodium carbonate and evaporate to dryness, finishing the process on the water-bath, to prevent the conversion of the urea to biuret (See Ex. 214). Extract the residue with strong alcohol or acetone. The alcohol or acetone solution is carefully evaporated to dryness, and the urea crystallises out.

CHAPTER XII.

DETECTION OF SUBSTANCES OF PHYSIOLOGICAL INTEREST.

A. Fluids.

1. Neutralise a considerable portion, and evaporate it to dryness, completing the process on a water bath to prevent charring. This evaporation to dryness is only necessary in tests for such substances as urea and the sugars. If these are known to be absent it can be omitted. It is as well to start this evaporation as soon as possible, as it takes a considerable time. Neutralisation is necessary to obviate any chemical changes produced by boiling acids or alkalies.

2. Note any characteristic smell of urine, bile, etc.

3. Note the colour and appearance of the fluid: opalescence suggests starch, glycogen, or certain protein solutions; coloured fluids suggest bile, blood or urine.

4. Note the reaction to litmus. An acid reaction excludes the presence of mucin, nucleo-proteins, caseinogen, and usually, of uric acid; an alkaline reaction usually excludes earthy phosphates.

5. Sprinkle some flowers of sulphur on the surface of a portion of the fluid contained in a beaker. If the particles fall through the surface, bile salts are present (Ex. 195).

6. If the fluid is brown or green, apply Gmelin's test (Ex. 192), or Huppert's test (Ex. 193) for bile pigments.

7. If the fluid is red or brown, examine for blood-pigments and derivatives by Table D.

8. If there are any reasons for suspecting the presence of ferments, examine by Table E. If none of the colour reactions for proteins are obtained, ferments are probably absent.

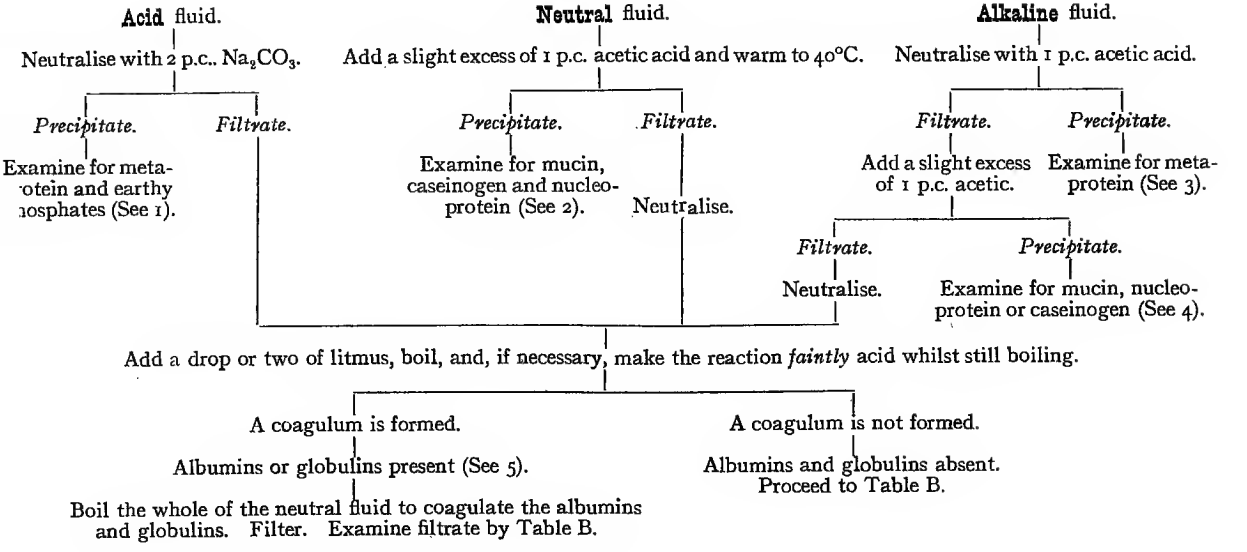
9. Examine for proteins by Millon's and the xantho-proteic reactions (Exs. 1 and 2). If they are present, proceed as directed in Table A. If they are absent, proceed to Table C.

The figures in parenthesis in Table A refer to the notes that follow the table.

10. Test for uric acid if the fluid is alkaline, neutral, or only faintly acid. Acidify with a drop or two of strong hydrochloric acid; uric acid may separate out as a crystalline powder. Make another portion of the solution alkaline with ammonia, saturate with NH_4Cl and apply the murexide reaction to the precipitate thus obtained (Ex. 217).

11. If the fluid be alkaline, treat a little with a solution of calcium chloride. A white curdy precipitate indicates the presence of soaps. (Their presence should be confirmed by the methods given in Ex. 111).

TABLE A.


Acid fluid.
Neutralise with 2 p.c.. Na_2CO_3.
Precipitate.
Filtrate.
Examine for meta-
otein and earthy
iosphates (See 1).
Neutral fluid.
Add a slight excess of 1 p.c. acetic acid and warm to 40°C.
Precipitate.
Filtrate.
Examine for mucin, caseinogen and nucleo-protein (See 2).
Neutralise.
Alkaline fluid.
Neutralise with 1 p.c. acetic acid.
Filtrate.
Precipitate.
Add a slight excess of 1 p.c. acetic.
Examine for meta-protein (See 3).
Filtrate.
Precipitate.
Neutralise.
Examine for mucin, nucleo-protein or caseinogen (See 4).
Add a drop or two of litmus, boil, and, if necessary, make the reaction *faintly* acid whilst still boiling.
A coagulum is formed.
Albumins or globulins present (See 5).
Boil the whole of the neutral fluid to coagulate the albumins and globulins. Filter. Examine filtrate by Table B.
A coagulum is not formed.
Albumins and globulins absent.
Proceed to Table B.

Notes to Table A.

1. Neutralise a small portion of the acid fluid, and then add a few drops of NaOH. If the precipitate entirely dissolves in the alkali, *meta-protein* is present and earthy phosphates are absent. If any precipitate is left when the NaOH is added, *earthy phosphates* are present. Confirm by filtering, dissolving the precipitate in HNO_3 and boiling with ammonium molybdate. If phosphates are present proceed as follows: the precipitate obtained by neutralisation is treated on the paper with 5 c.c. of water to which two or three drops of NaOH have been added. The meta-protein dissolves, the phosphates do not. Filter and neutralise the filtrate very carefully with dilute acid. A precipitate on neutralisation indicates *meta-protein*.

2. If bile-salts have been found to be present from the preliminary tests, this precipitate may consist of bile-acids with a protein (See Ex. 200) and can be neglected. If bile-salts are absent, treat the precipitate with an excess of strong acetic acid. If the precipitate does not dissolve it is *mucin*. If it dissolves to a rather opalescent solution it is caseinogen or nucleo-protein. These two proteins cannot be readily distinguished, but the presence of one of them can be confirmed by fusing the precipitate with fusion mixture and testing for phosphates (Ex. 44).

3. This precipitate may contain some mucin, caseinogen, or nucleo-protein. Great care should be taken to make the solution exactly neutral. Treat the solution with 5 c.c. of 1 p.c. acetic acid. It will entirely dissolve if it is *meta-protein*. Boil the solution with Millon's reagent: a red colour confirms the presence of the protein. It must not be forgotten that acetic acid will precipitate uric acid from an alkaline solution of urates, and fatty acid from an alkaline soap solution. Neither of these substances however will redissolve in an excess of dilute acid.

4. This precipitate should be examined by the method given in (2) above.

5. To differentiate between globulins and albumins proceed as follows:—

(*a*) Treat the neutral solution (free from meta-proteins, etc.) with an equal volume of saturated ammonium sulpbate solution. If a precipitate is not obtained, globulins are absent. Albumins are therefore present and can be confirmed by obtaining a coagulum on boiling the filtrate. A precipitate on half-saturation might consist of globulin, albumin, albumose, starch, etc.

(*b*) Dilute the neutral solution with about five times its volume of distilled water and add 1 p.c. acetic acid drop by drop. The formation of a cloudy precipitate either on dilution or the subsequent slight acidification indicates globulin. This test will not succeed if there is an excess of salt present, or if the amount of globulin is small. It is particularly applicable to myosin solutions.

TABLE B.

The fluid is now neutral or very faintly acid. All proteins except albumoses, peptones and gelatin have been removed (the fluid will be referred to as fluid A).

Treat a small portion with sodium hydrate and a drop of 1 per cent. copper sulphate.

I. If a pink colour is not obtained, proceed to Table C.

II. If a pink colour is produced, albumoses, peptones or gelatin are present. Try the glyoxylic test and Millon's test. If both are negative or practically so, then gelatin is present and albumoses or peptones absent. If the two tests succeed, albumoses or peptones are present, and gelatin can be regarded as absent. Then proceed as follows: treat another portion with a few drops of strong acetic acid and a single drop of potassium ferrocyanide: a white cloudy precipitate that disappears on warming and reappears on cooling indicates *albumoses* (Ex. 49).

Saturate another portion of fluid A with solid ammonium sulphate, by boiling with the solid salt in a test-tube, and filter. Treat the filtrate with **twice its volume of 40 per cent. sodium hydrate**

and a single drop of copper sulphate: a pink colour shows the presence of *peptone*. The precipitate, if any, should be washed with saturated $(NH_4)_2SO_4$, dissolved in a little hot water and the biuret test for albumoses applied to the solution.

III. If albumoses or peptones are present, test fluid A for starch, glycogen and dextrin (Table C), but use fluid B (see below) for the remaining tests.

Preparation of fluid B. The residue left after evaporation of the original solution, is rubbed up with 95 per cent. alcohol and warmed with this on a water bath for a short time. The alcohol is then filtered off, and evaporated to complete dryness on a water bath. The solution of the residue in a small amount of water is fluid B.

TABLE C.

(*a*) To a small portion, which has been acidified if necessary with acetic acid, add diluted iodine drop by drop, until an excess has been added. If a pure blue colour is obtained at any stage of the addition of iodine, starch is present. If a purple or brown colour is produced and the fluid is quite clear, erythro-dextrin is present and glycogen absent. If a blue colour is produced, or if the fluid is opalescent, proceed as follows:

To a portion of fluid A add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, and filter after about ten minutes through a dry paper.

Precipitate. Scrape off the paper, dissolve in a little hot water, cool and add a drop of iodine. A blue colour shows the presence of *starch.*	*Filtrate.* To a small portion add a drop or two of iodine. If a reddish or purple colour is produced, glycogen or dextrin is present. If the fluid is opalescent, *glycogen* is present. Saturate the remainder with $(NH_4)_2SO_4$ and filter.
	Precipitate. Neglect. / *Filtrate.* Add a drop of diluted iodine, a red-brown colour shows the presence of *erythro-dextrin.*

(*b*) Apply Fehling's test for *reducing sugars* (Ex. 63). It must be noted that the test will not succeed in the presence of any considerable amount of ammonium salts: that the reaction must be neutral or alkaline: that it is very much obscured by the presence of proteins, which must be removed if necessary.

Apply the naphthol test for *cane sugar* (Ex. 73). If reducing sugars and the polysaccharides are absent, test for cane sugar by boiling with acids, etc. (Ex. 72).

Treat some of the solution with sodium hypobromite. An evolution of gas indicates the presence of urea, an ammonium salt or amido-compounds. If there is any gas formation, attempt to obtain crystals of *urea nitrate* by adding a drop of nitric acid to a concentrated aqueous solution of the alcohol soluble substances.

Apply Oliver's test (Ex. 200), or Pettenkofer's test (Ex. 194) for *bile salts.*

TABLE D.

Examine the solution spectroscopically: gradually dilute the solution, noting the spectrum at all stages of dilution.

Take the reaction of the undiluted fluid to litmus paper, washing the surplus off the paper with a stream of distilled water, if you are unable to note the reaction directly.

If the fluid is neutral or alkaline, treat it with Stokes' fluid or warm it with ammonium sulphide, and note whether the spectrum is altered by reduction. This should be done after various dilutions of the original solution.

- Fluid red
  - Acid—*Acid haematoporphyrin*, two bands (Ex. 187).
  - Neutral — Dilute till two bands are well seen and then reduce.
    - *Oxy-haemoglobin*, the two bands merge into one faint band (Ex. 172).
    - *CO-haemoglobin*, the two bands are unaltered (Ex. 175).
  - Alkaline
    - *Alkaline haematoporphyrin*, four bands, converted to acid haematoporphyrin by strong acids (Exs. 187 and 188).
    - *Haemochromogen*, two bands in green, one much more distinct than the other, unaffected by reducing reagents (Ex. 186).
- Fluid Brown
  - Acid—*Acid haematin*, band in red (Ex. 182).
  - Neutral. *Methaemoglobin*, band in red: gives spectrum of oxy-haemoglobin and then of reduced haemoglobin if reduced (Exs. 179 and 180).
  - Alkaline
    - *Alkaline haematoporphyrin*—four bands (Ex. 188).
    - *Alkaline haematin*, faint band in red, converted to haemochromogen by reducing reagents (Exs. 184, 186).

Table E.

Take the reaction of the fluid to litmus.

I. Markedly *acid*.

Examine for pepsin (Exs. 149 or 147 A and D).

Neutralise very carefully, and examine for rennin (Exs. 125 and 126).

II. Very faintly acid, *neutral* or very faintly alkaline.

Examine for ptyalin (Exs. 140 and 141).

Examine for rennin (Exs. 125 and 126).

Examine for trypsin (Ex. 151).

Examine for pepsin (Exs. 149 or 147).

III. Markedly *alkaline*.

Examine for trypsin (Ex. 151).

Examine for steapsin (Ex. 106).

Perform control experiments in all cases (See Ex. 105).

A few special hints on the examination of physiological fluids.

1. It is impossible to obtain a heat coagulum of albumin or globulin in an acid or alkaline fluid. The reaction must be *neutral*, or only very faintly acid.

2. A little litmus solution in the fluid does no harm, and often reminds one that the reaction changes after boiling (due to the evolution of CO_2).

3. In testing for peptones after removing the albumoses by saturation with ammonium sulphate, the biuret test only succeeds if you use at least two volumes of 40 per cent soda. The test will not be obtained with the ordinary 5 per cent. soda.

4. Gelatin reacts very much like the albumoses, except that it does not yield the glyoxylic reaction.

5. It is impossible to obtain Fehling's test for the reducing sugars in the presence of ammonia or ammonium salts.

6. The sugars only reduce in an alkaline medium. If the fluid you are testing be acid, you must neutralise it before boiling with the Fehling's solution.

7. In testing for cane sugar do not forget that starch and the dextrins are hydrolysed to dextrose by boiling acids. But whereas cane sugar is hydrolysed very easily, starch, etc., are only slowly acted on.

8. Starch, glycogen and the erythro-dextrins do not give any colour with iodine solutions, if the reaction of the fluid be alkaline. If this be the case, make the reaction acid with acetic acid.

9. The proteins interfere with the iodine tests for these substances, and should therefore be removed as far as possible before testing for the polysaccharides.

10. Fat is insoluble in water, so do not waste time in testing an ordinary solution for fats.

11. The only reliable test for urea is to obtain crystals of the nitrate or oxalate. In this connection it must be remembered that urea is soluble in alcohol, and can thus be separated from the proteins and other substances that are likely to interfere with crystal formation.

12. Ammonium chloride is a very valuable reagent in testing for uric acid or urates. The only other physiological substance precipitated by it is soap.

13. Never omit "control" tests when investigating the ferment action of a solution.

14. Use "carmine fibrin" in testing for pepsin; never when testing for trypsin.

15. In testing solutions for pigments, examine spectroscopically in various dilutions. Note the reaction of the fluid; it is no good looking for oxy-haemoglobin in a markedly acid solution.

B. *Solids.*

1. Examine a little microscopically, both dry and with the addition of a drop of water. Look for starch grains, crystals of urea, uric acid, urates, leucin, tyrosin, cholesterin, and haemin scales.

2. Heat a small amount of the solid in a dry tube, at first gently and then more strongly.

(*a*) If sublimation takes place and an odour of amylamin is given off, leucin is present.

(*b*) If sublimation takes place and a strong smell of ammonia is evolved, urea is indicated.

(*c*) A smell of phenol and nitro-benzol indicates tyrosin.

(*d*) A smell of burning feathers indicates proteins, gelatin, etc.

(*e*) A smell of acrolein indicates fats.

3. Boil some of the solid with a small amount of water in a tube, cool under the tap. If gelatin is present, the solution will set to a jelly. (Starch, if present, may form a thick paste, which may be confused with the clean jelly given by gelatin. If the tube be subsequently placed in boiling water, gelatin becomes quite limpid, whilst starch remains thick).

4. If the solid is of a dark brown or red colour, boil a portion with dilute alkali, filter, heat the filtrate with Stokes' fluid or $(NH_4)_2S$, and examine for the spectrum of haemochromogen (Ex. 186). If this is obtained, the solid contains dried blood, or haematin. Confirm by obtaining haemin crystals (Ex. 189).

Analysis of a Solid for substances of Physiological Interest.

Heat a considerable amount of the solid with strong alcohol on a water bath, stirring well for some time. Remove the dish from the bath, and filter the alcohol into a dry vessel. Extract the residue once more with alcohol, filter and mix the alcohols.

<table>
<tr><td colspan="2">Alcoholic Solution. Evaporate to dryness on the water bath. Add 5 c.c. of water, heat and stir.</td><td colspan="4">Residue insoluble in alcohol. Treat with water, warm to about 40°C., and add acetic acid till the reaction is just acid. Cool and filter.</td></tr>
<tr><td>Residue. May contain fats, fatty acids or cholesterin.</td><td>Aqueous Solution. May contain urea, reducing sugars, cane sugar, bile-salts, soaps.</td><td>Solution. Test for albumoses and peptones (Table B). Glycogen and dextrin Earthy phosphates. (Table C).</td><td colspan="3">Residue. Treat with 2 per cent. Na_2CO_3, shake well and filter.</td></tr>
<tr><td colspan="3"></td><td>Solution. Test for urates (by Schiff's test) (Ex. 220), nucleo-proteins and caseinogen (Table A).</td><td colspan="2">Residue. Boil with water and filter.</td></tr>
<tr><td colspan="4"></td><td>Solution. Test for starch and urates.</td><td>Residue. Test for coagulated proteins.</td></tr>
</table>

APPENDIX.

1 grain = ·0648 gramme.
1 ounce = 437·5 grains = 28·3595 grammes.
1 lb. = 16 oz. = 7000 grains = 453·5925 grammes.
1 gramme = 15·432 grains.
1 kilogramme = 1000 grammes = 2 lb. $3\frac{1}{4}$ ozs. (approximately).
1 minim = ·059 c.c.
1 fluid drachm = 60 minims = 3·55 c.c.
1 fluid ounce = 8 fluid drachms = 28·4 c.c.
1 pint = 20 fluid ozs. = 567·9 c.c.
1 c.c. = 16·9 minims.
1 litre = 1000 c.c. = 35·2 fluid ozs.
1 gallon = 8 pints = 4·548 litres.
1 inch = 2·54 cm.
1 foot = 30·48 cm.
1 yard = 91·44 cm.
1 cm. = ·39 in.
1 metre = 39·37 in.

Conversions.

To convert grammes per 100 c.c. into grains per ounce, multiply by 4·375.

To convert grammes into ounces, multiply by 10 and divide by 284.

To convert litres into pints, multiply by 88 and divide by 50.

To convert kilos into pounds, multiply by 1000 and divide by 454.

To convert degrees Fahrenheit into degrees Centigrade, subtract 32, multiply the remainder by 5, and divide the result by 9.

To convert Centigrade into Fahrenheit, multiply by 9, divide by 5, and add 32.

Tension of Aqueous Vapour in millimetres of mercury from 8° to 25°C.

8°	8·0	14°	11·9	20°	17·4
9°	8·6	15°	12·7	21°	18·5
10°	9·2	16°	13·5	22°	19·7
11°	9·8	17°	14·4	23°	20·9
12°	10·5	18°	15·4	24°	22·2
13°	11·2	19°	16·3	25°	23·5

Approximate Atomic Weights of some of the Elements.

Barium	Ba	137	Hydrogen	H	1	Potassium	K 39
Bismuth	Bi	208	Iodine	I	127	Silver	Ag 108
Bromine	Br	80	Magnesium	Mg	24	Sodium	Na 23
Calcium	Ca	40	Manganese	Mn	55	Sulphur	S 32
Carbon	C	12	Mercury	Hg	200	Tin	Sn 119
Chlorine	Cl	35·5	Nitrogen	N	14	Uranium	U 240
Copper	Cu	63	Oxygen	O	16	Zinc	Zn 65
Fluorine	F	19	Phosphorus	P	31		

Phenyl-glucosazone (Ex. 66).

Fine yellow needles in fan-shaped aggregates, sheaves or crosses.

Phenyl-lactosazone (Ex. 68).

Ovoid or spherical clusters of fine yellow needles.

Phenyl-maltosazone (Ex. 70).

Broad yellow plates, either singly or arranged in spherical clusters.

Potato Starch (Ex. 75).

Ovoid or elliptical grains, with concentric markings and an eccentric hilum.

Wheat Starch (Ex. 133).

Small circular grains with a cetral hilum.

Tyrosin (Ex. 152).

Feathery masses and sheaves of fine white needles.

Leucin (Ex. 152).

Rounded cones or balls with a radiating striation.

Oxyhaemoglobin (dog.) (Ex. 167).
Thin rhombic prisms.

Haemin (Teichmann's Crystals, Ex. 189).
Brown rhombic prisms.

Cholesterin (Ex. 196).
Rhombic plates, often incomplete.

Urea crystallised from acetone (Ex. 205).
Long four-sided prisms, or fine needles.

Urea, crystallised from alcohol (Ex. 206).
Irregular branching masses.

Urea Oxalate (Ex. 203).

Long thin flat crystals, often in bundles. Rhombic prisms.

Urea Nitrate (Ex. 204).

Octahedral, lozenge-shaped, or hexagonal plates, striated or imbricated.

Uric Acid (Exs. 216, 237, 256).

(*a*) Rhombic plates.

(*b*) Irregular forms, such as dumb-bells, whet-stones, butcher-trays, stars and crosses.

Urinary Sediments (Ex. 256).

(*a*) Urates (spheres wiih projecting spines).

(*b*) Calcium oxalate (envelopes or dumb-bells).

(*c*) Calcium hydrogen phosphate (stellar phosphate).

(*d*) Ammonium-magnesium phosphate (triple phosphate), prisms (coffin lids) or feathery stars.

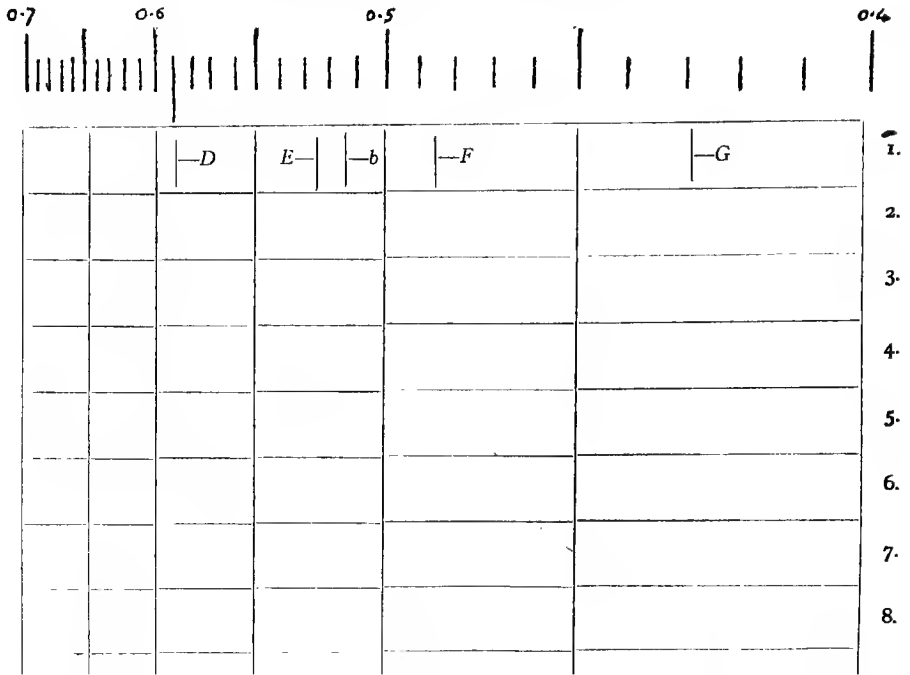
(*e*) Calcium carbonate, dumb-bells or spheres with radiating structure.

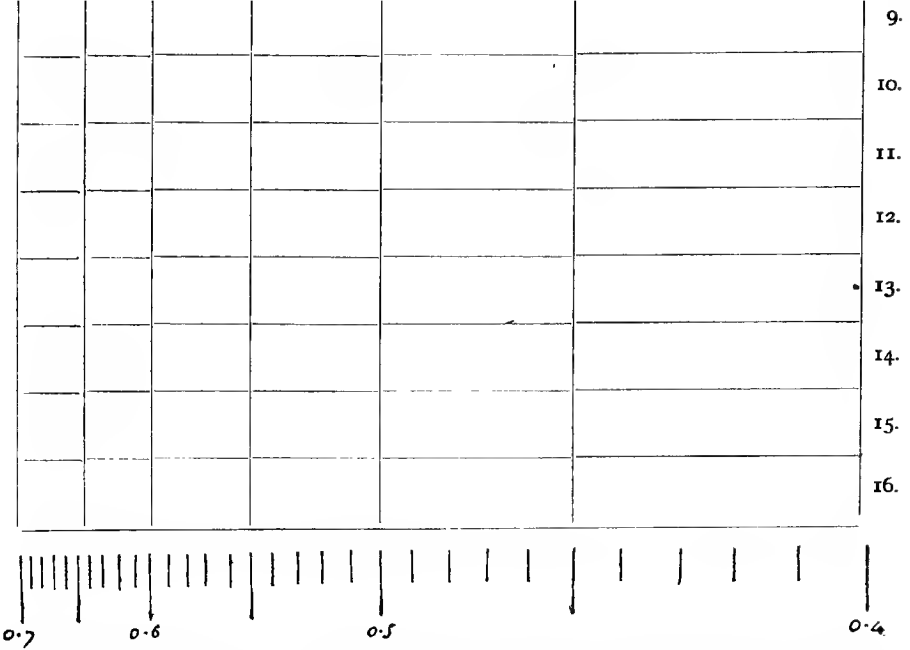
Sodium Urate (Ex. 222).

Spheres with thin projecting spines.

CHART FOR
RECORDING THE ABSORPTION-SPECTRA
OF PIGMENT SOLUTIONS.

CHART FOR RECORDING THE ABSORPTION-SPECTRA OF PIGMENT SOLUTIONS.





1. Fraunhofer's lines.
2. Oxy-haemoglobin (dilute).
3. Oxy-haemoglobin (medium).
4. Oxy-haemoglobin (strong).
5. Reduced haemoglobin.
6. CO-haemoglobin.
7. Methaemoglobin (strong).
8. Methaemoglobin (dilute).
9. Acid haematin.
10. Acid haematin in ether.
11. Alkaline haematin.
12. Haemochromogen.
13. Haematoporphyrin (Acid).
14. Haematoporphyrin (Alkaline).
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16. Pettenkofer's reaction.

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Printed at the Eagle Printing Works (Cambridge), Ltd.